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N IMPROVED RADIATION INTEGRATOR FOR BIOLOGICAL USE

BY D. L. GUNN, R. L. KIRK AND J. A. H. WATERHOUSE

(*Received* 1 *March* 1945)

(With Three Text-figures)

I. INTRODUCTION

ne variation in the sun's radiation is the principal ıse of variations in temperature in the field, with ny familiar consequences; but the measurement a single temperature gives a poor indication of what ıer temperatures in the neighbourhood are likely be. For example, the temperature of the body of insect exposed to full sunshine may be much ıher than the air; this is known in both alpine rüger, 1931) and in tropical conditions (Buxton, 24; Gunn, 1942). The importance of measuring intensity of the sun's radiation directly has been ently urged by Buxton (1926, 1927). Unfor- ıately, a convenient instrument for doing so in the d does not exist. It is not enough to measure light ensity, for the readings given by a photometer ɔend on the weight given to the various wave bands the particular type of instrument used. No photo- ter gives due weight to the short infra-red between nd 3 μ. A considerable proportion of the heat from sun, however, comes in this wave band. More- r, the proportion of the total radiation in this ion which reaches the earth's surface varies con- erably according to solar altitude and atmospheric ditions. Consequently, if you want to know the ting effect of the sun, you have to convert all the ident radiation into heat and measure that.

Most pyrheliometers are delicate and expensive ctrical instruments requiring skilled use. They do lend themselves readily, therefore, to use in the d or in remote localities—the use of the Kalitin onometer by Strelnikov (1936) in the Terek river ion is exceptional—with the result that no large eral body of knowledge exists about the distribu- ı of intensities of radiation over the earth's surface ɔugh the seasons of the year and through the daily le. The acquisition of such knowledge depends on availability of an inexpensive, robust and de- dable instrument for making measurements.

The type of instrument here described has de- ɔped from W. E. Wilson's 'Radio Integrator' iffiths, 1923). The principle of it is that radiation on a black body, so that it is all converted into t, the heat is transferred to a volatile liquid and sformed into latent heat of evaporation, the ɔur is condensed and its quantity is measured. If e processes can be carried out at 100 % efficiency, without the leakage of heat, an exact measure of radiation can be obtained, the total distillate indicating the total amount of radiation.

II. PREVIOUS MODELS

The original Wilson 'Radio Integrator' is all glass, with some black material distributed through the volatile liquid, believed to be alcohol* (Fig. 1). Kennedy (1939) obtained distillates of over 60 c.c./day with this instrument in the Anglo-Egyptian Sudan, but we have been able to get rates of only fractions of 1 c.c./hr. under laboratory conditions. The bulb receiving the radiation is fully exposed to the air, so that in a given intensity of radiation its temperature would be much influenced by the speed and temperature of the wind. Consequently it could never give an uncomplicated measure of radiation.

For this reason, Buxton (1926, 1927) designed an improved model in which the radiation receiver is enclosed in an evacuated glass jacket (Fig. 2 *a*). Buxton obtained some valuable results with this in Samoa, but found it difficult to standardize the construction of the apparatus, and various instruments of the same design gave mutually inconsistent readings. The efficiency of this instrument is considerably lower than the estimated 38 %, for there are errors in allowing for the exposed black area and in the latent heat of unit volume of alcohol. A figure of 4 % seems to be somewhat nearer the truth. Clearly, proportionately slight variations in the 96 % of heat loss will produce relatively large variations in the distillate, so that the instrument cannot be very accurate.

Buxton considered that the difference between his instruments might be due to variation in the length and diameter of the route followed by the alcohol vapour. He designed a model† (Fig. 2 *b*) in which this could be standardized and regretted that it was then technically impossible to use a black metal receiver instead of a glass one. His improved model has not been used in the field.

Consideration of the situation inside the radiation

* We are indebted to the Director of the Meteorological Office for the loan of this instrument.

† We are indebted to Prof. P. A. Buxton, F.R.S., for the loan of both of his patterns of instrument.

receiver and the alcohol condenser indicates that it is important for this space to be evacuated as highly as possible. If air is present, when the receiver is heated some alcohol will evaporate and the pressure will rise throughout the system; but, broadly speaking, the alcohol vapour will at first simply drive air out of the receiver and the rise of pressure in the condenser will be mainly due to the compression of air in it and not to the arrival of alcohol vapour. Alcohol will arrive slowly, after diffusion through the air. In the receiver the rise of alcohol vapour pressure will restrict further evaporation, insufficient heat will be transformed into latent heat and the temperature of the receiver will rise unduly. This, in turn, will lead to heat losses by conduction through the glass and by re-radiation. If, however, all air is removed, a rise of vapour pressure in the receiver will tend to lead to a practically instantaneous rise in alcohol pressure in the condenser, immediately offset by condensation, so that heat reaching the receiver can be rapidly removed and a considerable temperature rise avoided.

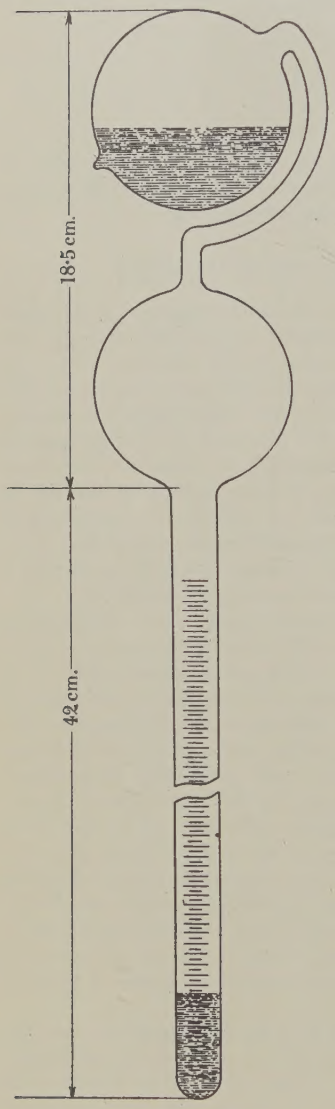

Fig. 1.

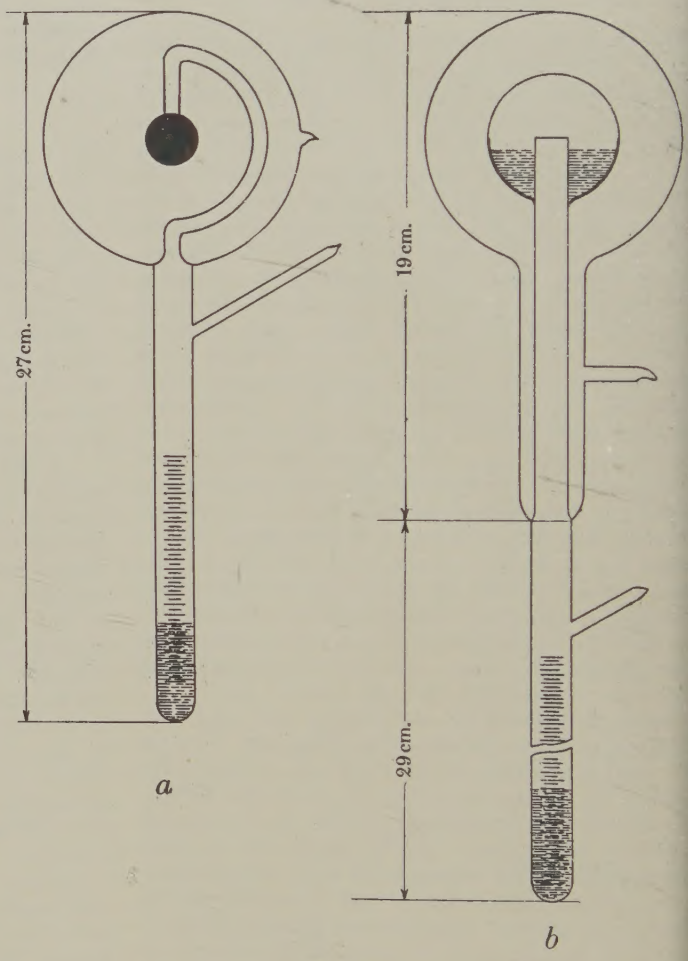

Fig. 2.

III. AN IMPROVED RADIATION INTEGRATOR

Our instruments incorporate the evacuated jacket of Buxton's first instrument and the shortened diffusion route of his improved model. The receiver is a hollow

copper sphere blackened on the outer surface, so that radiation falling on the top is converted into heat and rapidly conducted to the liquid below. The air is removed from the condenser. In some of the instruments a short piece of copper tubing has been inserted at the top of the condenser column (Fig. 3 *a*) to allow rapid escape of latent heat of condensation. Both alcohol and water have been used as the volatile liquid to give instruments of different sensitivity.

IV. CONSTRUCTION OF THE APPARATUS

The incorporation of copper in a glass apparatus which has to be vacuum-tight requires highly skilled construction. The principal difficulties which have to be overcome arise from the necessity of avoiding damage to one joint—glass to glass, glass to copper, or copper to copper—when making another. The details are here given in the order in which they were carried out.

The copper sphere, which weighed 20 g. and was 5·08 cm. in diameter, was supplied as two hemispheres, mated and ready for soldering together.* The lower half was drilled axially to receive a shouldered sleeve, which was then fixed with hard solder (Fig. 3 *b*). The copper tube, to which a piece of glass tubing had already been fixed, was then hard soldered to the top of the sleeve in such a way that no copper tube showed outside the sphere. The upper hemisphere was then soldered into position and the assembly tested for air-tightness.

A cup-shaped piece of glass was fused on to a piece of the same kind of tube ready to receive the glass envelope. This tube was then fused at one end to the glass tube projecting from the copper sphere and, at the other end, either to the condenser tubing or to a double-ended copper-glass seal. At this stage the copper sphere was heavily coated with lamp black from a coal-gas and benzene flame, such as is used for smoking kymograph papers. It is important, if high absorption is required over the entire spectral range from 0·4 to 3·0 μ, not to paint on the blackening material and not to use anything but lamp black deposited from a flame. The copper sphere was then inserted into the outer envelope—a 600 c.c. Chance's Hysil CO_2 titration flask with the rim cut off and the flat bottom blown out in an attempt to complete the spherical shape.† The flask was fused on to the cup prepared for it, and the whole apparatus oven heated at 400° C. for 30 min. to release adsorbed gases, while a high-vacuum pump evacuated the envelope through a side tube attached below the level of the receiver. The envelope was sealed off when a steady vacuum of 10^{-5} mm. Hg had been reached. The condenser (Chance's Veridia Hysil of very uniform bore) was next fused into position.

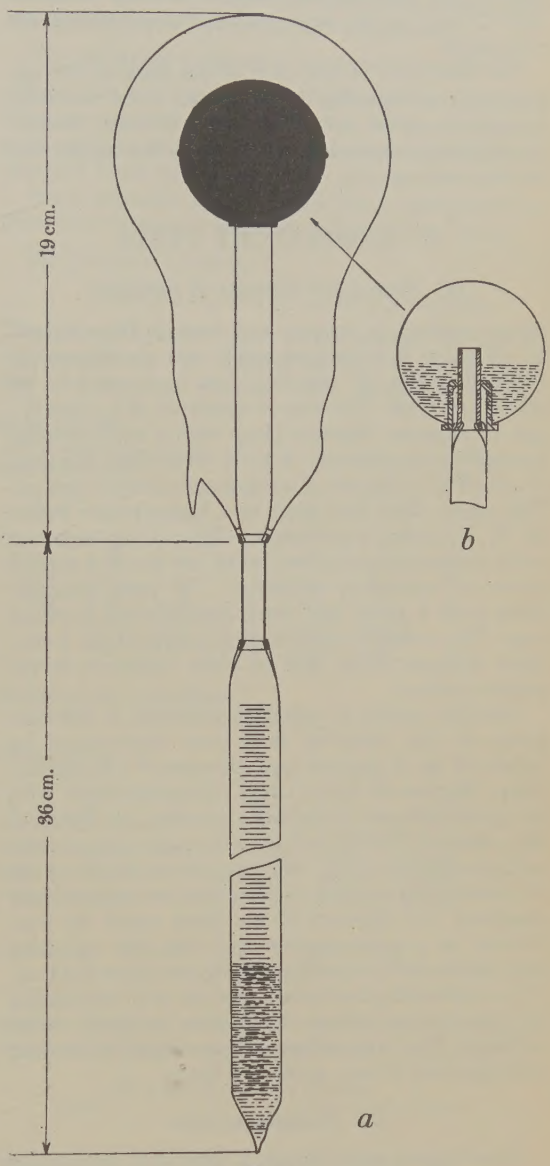

Fig. 3.

The final operation is to put in the alcohol and seal up the condenser. This was also done by high-vacuum technique, the whole of the sealed system, including the alcohol, being cleared of air before the alcohol was distilled from a reservoir into the condenser. The condenser was then sealed off in the blowpipe, enough alcohol having been left in the apparatus to come up to the first undistorted gradua-

* We are indebted to Mr John Levick, of Levick and Sons Ltd., Aston, Birmingham, for the gift of a number of these spun spheres of different weights and sizes.

† Chance's Hysil was chosen because of its remarkably good transmission up to nearly the limit of the solar spectrum at the earth's surface. For a specimen 2·5 mm. thick the percentage transmission is 90 between 0·5 and 2·0 μ, 80 at 2·5 μ and 20 at 2·7 μ.

tion on the condenser when the receiver was holding as much as it could in the operating position. The same filling procedure was followed when the alcohol was replaced by water, with the exception that with water-filled radiometers, 0·09 g. of soap dried at 100° C. was put into each receiver before starting the evacuation.

The inclusion of the neck of the flask in the apparatus is undesirable, but it appears to be necessary in order to avoid overheating the radiation receiver during manufacture and so oxidizing the copper and causing scaling.

V. LABORATORY TESTS

(*a*) *Source and intensity of radiation*

Since sunshine is too rare and weak in Birmingham in winter to provide reasonable test conditions, an artificial source of radiation has been used in all laboratory tests. The source consisted of a 1000 W., 230 V. tungsten filament lamp with a white enamel hemispherical reflector. A 5 cm. water filter was used to cut off all radiation of wave-length longer than 3 μ. The water filter was kept at a temperature below 30° C. by using a continuous flow of tap water at room temperature, so that it did not act as a serious source of secondary radiation. The peak intensity from such a lamp and filter combination is about 0·9 μ. The radiation distribution is, therefore, somewhat different from that of solar radiation at the earth's surface.

Absolute values of radiation intensity at different points in the radiation field were determined by means of an Ångström pyrheliometer* (Ångström, 1894; Hogben & Kirk, 1944). Measurements with the pyrheliometer in the same position, on different days, showed that the radiation intensity varied about a mean value by $\pm 7\%$. By using the mean of a series of values it was possible to show that, for approximate purposes, the filament of the lamp could be considered as a point source and that the radiation intensity varied inversely as the square of the distance. This relationship has been made use of in calculating all intermediate values of radiation intensity when required. The range of intensity available for testing purposes was 0·1–0·7 g.cal./cm.^{2}/min.

(*b*) *Preliminary tests*

Experiments with Buxton's improved instrument encouraged the belief that evacuation of the condenser was an important factor in the performance of the Integrator. Distillates obtained when the condenser of his improved instrument was roughly evacuated were twice as great as when there was air in the condenser at atmospheric pressure. The first instrument to be constructed, therefore, was evacuated to 10^{-5} mm. Hg, and although the copper receiver was unblackened, distillates of 2·5 c.c./hr. were obtained at a radiation intensity of 0·7 g.cal./cm.^{2}/min. Quite obviously the efficiency of the instrument also depends on the rate at which latent heat of condensation can be transferred from the condenser to the surrounding air. Using thermocouples dipping into mercury contained in plasticene cups attached to the outside of the condenser tube it was possible to determine the rise in temperature of various parts of the condenser when vigorous distillation was taking place. At a distillation rate of 5 c.c./hr. the temperature of the upper section of the condenser tube was 11° C. above that of the surrounding air. Two possibilities presented themselves for preventing this large rise in temperature. The first was to increase the area of glass on which the condensation could take place by the inclusion of a glass globe at the top of the condenser column. The second was to increase the rate of transfer of heat from the condenser to the air by including in the condenser column a section of material of high thermal conductivity. Consideration of the mathematics of the thermal conductivities of copper and glass suggested that, to be as effective as a short piece of copper tube let into the condenser column, the glass globe would have to be so large as to make the whole apparatus unwieldy. The second alternative was adopted, therefore, when constructing some of the final instruments.

(*c*) *Calibration of the improved models of the radiation integrator*

Table 1 gives a list of the instruments finally made, with an indication of their special characteristics.

Table 1

Instrument no.	Volatile liquid	Form of condenser
1	Water	Copper-glass. Condenser contains air at atmospheric pressure
2	Water	Copper-glass. Condenser evacuated
3	Water	Copper-glass. Condenser evacuated
4	Water	All glass. Condenser evacuated
5	Water	All glass. Condenser evacuated
6	Alcohol	Copper-glass. Condenser evacuated
7	Alcohol	Copper-glass. Condenser evacuated
8	Alcohol	Copper-glass. Condenser evacuated
9	Alcohol	All glass. Condenser evacuated
10	Alcohol	All glass. Condenser evacuated

These instruments have been tested under artificial radiation conditions and the results are tabulated in Table 2. The values of distillate in c.c./hr. were obtained by plotting condenser reading against time and determining the slope of the resulting straight line when a steady rate of distillation had been reached (generally after about 30 min.). At first some diffi-

* We are indebted to Mr R. H. Knight, M.Sc., for the construction of this apparatus.

culty was met in using the instruments containing water due to the formation of droplets which refused to run smoothly down the side of the condenser column. The inclusion of the small amount of soap, already referred to, in the water in the condenser overcame this difficulty. The soap films which tend to form when the liquid is disturbed can easily be removed by gentle warming of the condenser and a sharp meniscus is obtained.

The instruments were tested close to the two extremes of radiation intensity available to us. Considerable differences occur when individual instruments are tested on different occasions, and between different instruments when tested together. Some possible causes of these differences could easily have been removed had the instruments not been urgently required for field work. For example, the glass envelopes were not spherical and of a uniform thickness, so that lens effects and reflexions could occur; there were in some cases spots of dirt on the inside of the glass; it is not certain that the copper receivers were all equally, evenly or adequately blackened. Moreover, there was a variation in the intensity of radiation, as shown by the pyrheliometer at one point at different times, presumably due to variations in mains voltage, and the intensity measurement could not be carried out at the same time and point in space as the radiometer test. It is clear, however, that since the instruments fall approximately in the same order in different tests it is possible to average the results for a number of tests and to work out a mean efficiency for each of the instruments.

The calculated mean efficiencies are given in Table 3. When a steady rate of distillation is reached, we may neglect the thermal capacity of the receiver and condenser systems. Then, if we assume perfect black-body absorption, the radiation received by a sphere from a point source at a distance d from the centre of the sphere is proportional to the solid angle subtended by the sphere at the source, i.e.

$$\text{the radiation received} \propto 2\pi\,(1-\sqrt{[1-a^2/d^2]}),$$

where a is the radius of the sphere. The tungsten filament was in the form of a circle, so that each point on it could be considered as a point source at the same distance from the centre of the sphere.

If the intensity measured by the pyrheliometer at distance d from the filament is I g.cal./cm.^{2}/min.

$$\text{the radiation received} = Id^2 \times 2\pi\,(1-\sqrt{[1-a^2/d^2]}) \text{ g.cal./min.}$$

The latent heat of pure ethyl alcohol at 40° C. (approx. temp. of copper sphere) = 218·7 cal./g. and the density of pure ethyl alcohol at 20° C. (approx. temp. of condenser) = 0·7876 g./c.c.; therefore the distillation in the condenser is given by

$$D_A = \frac{Id^2 \times 2\pi\,(1-\sqrt{[1-a^2/d^2]})}{218{\cdot}7 \times 0{\cdot}7876} \text{ c.c./min.}$$

Similarly for water the distillate is given by

$$D_W = \frac{Id^2 \times 2\pi\,(1-\sqrt{[1-a^2/d^2]})}{573{\cdot}7 \times 0{\cdot}9982} \text{ c.c./min.}$$

The value of a is 2·54 cm., and for a value of $I = 0{\cdot}7$ g.cal./cm.^{2}/min. $d = 27{\cdot}7$ cm., so that

$$D_A = 5{\cdot}031 \text{ c.c./hr.} \quad \text{and} \quad D_W = 1{\cdot}513 \text{ c.c./hr.}$$

The efficiency is obtained by dividing the observed distillate at a given radiation intensity by the appropriate calculated distillate.

Despite the uncertainties involved in calculations

Table 2. *Distillate in c.c./hr.*

Intensity of radiation g.cal./cm.^{2}/min.	Instrument no.									
	1	2	3	4	5	6	7	8	9	10
0·18	0·00	0·27	0·42	0·42	0·40	1·28	1·18	1·20	0·63	1·06
0·20	0·07	0·30	0·46	0·40	0·38	1·29	1·50	1·47	1·00	1·23
0·70	0·27	1·37	1·48	1·58	1·40	4·85	5·40	4·75	4·60	4·65
0·70	—	—	—	—	—	4·80	5·15	5·10	4·25	4·90
0·70	—	—	—	—	—	4·57	4·88	4·75	4·32	4·47

Table 3. *Ratio: observed distillate/calc. distillate.*

Intensity of radiation g.cal./cm.^{2}/min.	Instrument no.									
	1	2	3	4	5	6	7	8	9	10
0·18	—	0·694	1·079	1·079	1·028	0·989	0·912	0·928	0·487	0·819
0·20	0·162	0·695	1·066	0·927	0·881	0·899	1·046	1·025	0·697	0·856
0·70	0·178	0·905	0·978	1·044	0·925	0·942	1·022	0·970	0·873	0·928
Mean efficiency %	17·0	76·5	104·1	101·7	94·5	94·3	99·3	97·4	68·6	86·8

of this kind, it is probable that the overall efficiency of our instruments, with the exception of nos. 1, 2 and 9, exceeds 80%, and that in some of the instruments it is close to the theoretical maximum.

(*d*) *Direct comparison with other types of radiation integrator*

We have carried out a number of tests designed to compare directly the efficiency of our own instruments with other types of radiation integrator. Table 4 is the record of a number of such tests on the original Wilson 'Radio Integrator' and Prof. Buxton's first model and also his improved model with the condenser either evacuated or containing air at atmospheric pressure.

Table 4

Radiation intensity g.cal./cm.²/min.	Distillates in c.c./hr.				
	Wilson Integrator	Buxton no. 1	Buxton no. 2 (condenser evacuated)	Buxton no. 2 (condenser not evacuated)	Mean value for instruments nos. 5–10
0·7	0·14	0·017	0·12	0·02	4·70

VI. DISCUSSION

In an apparatus of the size and type under discussion it is still not possible to standardize completely all the factors which affect the final performance of the instrument. Such factors as the departure from sphericity of the glass envelope, the thickness and quality of the lamp-black deposit on the copper receiver, and, of particular importance in the case of the water instruments, the degree of chemical cleanliness of those parts of the apparatus in contact with the volatile liquid, will inevitably produce differences between instruments otherwise constructed with the greatest care. Bearing this in mind, the type of radiation integrator here described represents a considerable improvement on previous models. Under carefully controlled laboratory conditions neither the original Wilson instrument nor the Buxton instruments will satisfactorily measure low intensities of radiation unless exposed for long periods of time. Moreover, it is certain that in instruments with such a low overall efficiency, estimates of higher intensities of radiation are subject to considerable errors. This does not apply to anything like the same extent in instruments with efficiencies of the order of 80% or more.

The chief differences between the improved Buxton model and our own are the use of a copper receiver instead of a glass one, and the evacuation of the condenser system. Of the two alterations in design the latter would seem, from experimental results, to be far the more important. The inclusion of a copper section in the condenser column further improves the efficiency by about 10%. For general purposes, however, the inclusion of this refinement may not be advisable because it considerably increases the difficulties of construction and also because the exposed copper-glass seals are liable to fracture.

While, as yet, none of our instruments have been exposed to tropical sunshine,* it is possible to estimate roughly the amount of distillate which would be obtained under such conditions. At an intensity of 1·5 g.cal./cm.²/min. the alcohol instruments would distil about 10 c.c./hr. Changes in radiation intensity over half-hour periods can, therefore, be detected with ease if readings are taken every 5 min. The time necessary for the determination of intensities in the early hours of the morning and late afternoon will, of course, be somewhat longer, and since this is the time of the day when the intensity of solar radiation is changing most rapidly, the accuracy of the estimates will be correspondingly lower. It is obvious, however, that the systematic use of one of these alcohol instruments can yield valuable information about variations in intensity of solar radiation in localities where such measurements have hitherto been impossible.

The water instruments, on the other hand, would seem to be most satisfactory for determining the mean daily intensity of sunshine, and so elucidating the variations in this meteorological element at different periods of the year and at different places. Assuming a mean intensity of 0·6 g.cal./cm.²/min. one of the water instruments would distil about 15 c.c. during 12 hr. of sunshine.

The radiation integrator, together with a metal shield, has been fitted into a kind of suit-case and its total weight is then 12 lb. It can easily be carried by hand, can rapidly be set up in the operating position, and requires little attention while in use.

The most serious disadvantage of this type of instrument is its high thermal capacity. This means that it is incapable of detecting rapid fluctuations in radiation intensity, such as is caused, for example, by the presence of clouds. For some kinds of biological work, therefore, where such rapid fluctuations in intensity may be of importance, there does not seem to be any alternative to the use of a more delicate electrical pyrheliometer. The design of such an

* It is hoped to publish shortly some results of the field use of the new type of radiation integrator.

strument, robust enough to withstand transport d requiring the minimum of accessory apparatus, ust be elaborated in the future if every aspect of lar and terrestrial radiation of importance to the ologist is to be evaluated.

VII. SUMMARY

1. A new radiation integrator is described similar one previously described by Buxton.

2. Methods of construction are given in detail to cilitate the standardization of future instruments.

3. Instruments of different sensitivities have been nstructed by the use of either alcohol or water as e volatile liquid. The alcohol instruments can be used for determining changes in intensity over short periods of time; the water instruments are of most value for determining the mean daily intensity of sunshine.

4. The instruments have been calibrated against absolute measurements with a pyrheliometer. Compared with previous instruments of the same type a very much improved efficiency has been obtained.

5. The value of this type of instrument for work in the field is discussed; its limitations are briefly indicated.

Two of us (R. L. K. and J. A. H. W.) are indebted to the Rockefeller Foundation for a grant which we received during the course of this work.

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ON THE PENETRATION OF INSECTICIDES THROUGH THE INSECT CUTICLE

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(*Received* 6 *May* 1945)

(With Two Text-figures)

CONTENTS

INTRODUCTION

During work on the control of human pediculosis Craufurd-Benson & Macleod found that body lice, which normally have a high resistance to derris, were rapidly killed when high-boiling tar acids were added to the insecticide. A similar effect was observed in experiments with the sheep ked, *Melophagus ovinus*, when mixtures of cresylic acid and diphenylamine were applied either as a dusting powder with a china clay base, or as a liquid wash. It was suspected that a rapid penetration of the insecticide through the insect cuticle, induced by the presence of the solvent, might be responsible for this increase in efficiency. If the synergy, already found to exist between derris or diphenylamine on the one hand, and phenols on the other, could be extended to other insecticides and solvents, then an understanding of the factors governing the phenomenon would have considerable value in deciding the correct mode of presentation of a particular insecticide.

THE EFFECT OF ORGANIC SOLVENTS ON THE RATE OF PENETRATION OF DIPHENYLAMINE

As diphenylamine has a high solubility in the majority of organic solvents it was decided to use this insecticide in the investigation of the problem. A number of these solvents with widely differing physical properties were tested for insecticidal efficiency, both with and without the insecticide.

Powders containing solvent + insecticide were prepared in the following manner. A mixture of 10% ground diphenylamine and china clay was diluted with more china clay to give a concentration of 0·25% diphenylamine in the powder. To this, 1% by weight of solvent was added, and the powder then shaken mechanically for half an hour. In these proportions the diphenylamine was completely soluble in all the solvents tested. Control powders containing 1% by weight of solvent alone were prepared in a similar manner.

The test insect used in these experiments was the sheep ked, *M. ovinus*. A batch of five keds was placed in a corked specimen tube, liberally dusted with the powder under test and incubated at 30°C. Treated keds were observed under the binocular microscope at half-hourly or hourly intervals for a maximum period of 30 hr. or until they died. The criterion taken to indicate death was the final cessation of heart beat: in the ked the beating heart is readily visible through the dorsal integument of the abdomen. When making observations, each ked was removed temporarily from its tube and the powder lightly brushed from the dorsal surface of the abdomen, sufficient powder being placed initially in the tube to ensure a surplus with which the ked became recoated after each examination.

It was shown by Webb (1945*a*) that the susceptibility of keds to ground derris root increased in the presence of 5% carbon dioxide which, by stimulating respiration, caused greater quantities of the insecticide to be taken in through the spiracles. To determine the extent to which powders on a china clay base penetrate the spiracles, keds were dusted with a powder containing 0·25% diphenylamine without solvent, in air, and in a mixture of air and 5% carbon dioxide. As no difference in time of death of the keds was observed in the two series, it was assumed that the amount of diphenylamine entering the spiracles was negligible and that pene-

ation was taking place primarily through the external cuticle. Over a number of experiments with 25% diphenylamine alone in china clay, keds died 25–30 hr.

In Table 1 the results of the experiments with powders containing diphenylamine + solvent, and solvent alone, are shown. The time of death of keds for each powder is based on the findings of a number of trials. It is seen that the addition of a solvent to diphenylamine in some cases reduced the time of death from 25–30 hr. to as low as 1·5 hr., whereas in others little or no reduction was observed.

Some of the solvents when used alone were found to exert a lethal effect. With the exception of the treated with benzyl alcohol alone died in 21 hr., whereas those treated with benzyl alcohol + diphenylamine died in 2·5 hr. This shows that a reduction in the time of death of keds of the same order as that obtained on adding cresols or xylenol to diphenylamine can be obtained with a comparatively non-toxic solvent. It is not proposed, therefore, to consider the toxicity of the solvent in assessing the results obtained with mixtures of solvent and insecticide, except where the toxicity of the solvent alone approaches that of the mixture. An example of this is given by *o*-dichlorobenzene, where the effect of the solvent alone is as great as that of the mixture. Here, the solvent is not held to promote

Table 1. In vitro *experiments with diphenylamine*

Solvent	Time of death of keds in hours		Time in hours at which keds became immobile after treatment with solvent control
	Solvent + insecticide	Solvent control	
o-Cresol	1·5	10	1·0
m-Cresol	1·5	9	1·0
Xylenol	1·5	9	1·0
Benzyl alcohol	2·5	21	2·0
p-Cresol	2·5	9	1·0
Octyl alcohol	4	30	2·0
4-Methyl-cyclohexanol	5	30	2·0
Quinoline	6	27	2·0
Cyclohexanone	8	26	2·0
Diacetone alcohol	13	—	—
Cyclohexanol	17	21	2·0
Acetophenone	22	28	1·0
Benzonitrile	23	—	6·0
Aniline	24	—	1·0
Carbitol	24	26+	20
Dimethyl-aniline	27	—	1·0
Methyl benzoate	27	27	2·0
Castor oil	26+	—	—
Anisole	27+	—	—
o-Dichlorobenzene	21	19	3·0

N.B. Keds dusted with 0·25% diphenylamine die in 25–30 hr.

esols and xylenol, however, this was so slight that may, for practical purposes, be neglected. Even th these solvents, although their toxicities are latively high, they are considerably lower than ose observed when diphenylamine is present. most all the solvents used caused immobility of e ked in a comparatively short time (see Table 1), it in every case subsequent death, when it occurred, was protracted. In many instances keds again came active, even after a lapse of more than 5 hr.

It is doubtful to what extent toxicity of solvent fluences results obtained with mixtures. Although ds already suffering from cresol or xylenol poisong would probably be slightly less resistant to diphenylamine than unaffected keds, the reduction in e lethal dose of the latter due to the toxicity of the lvent is unlikely to be great. For instance, keds the rate of entry of diphenylamine through the cuticle and it is, therefore, placed lowest in order of efficacy in Table 1.

As certain organic solvents of diphenylamine, notably phenols and benzyl alcohol, when added to that insecticide greatly increase its rate of action, while others, such as dimethyl-aniline, methyl benzoate and anisole, appear to have little or no effect, it is clear that solubility of diphenylamine in the solvent is not the only factor involved in this process.

It is suggested that solvents may increase the rate of penetration of diphenylamine through the cuticle in one of two ways. First, the diphenylamine may be transported through the cuticle in solution in droplets or small molecular aggregates of the solvent, and, secondly, the presence of solvent may render the cuticle more permeable to the insecticide. As the

time taken for the solvent alone to penetrate the cuticle, i.e. the time in which the solvent causes immobility of the ked, bears no relationship whatsoever to the time of death when diphenylamine is present (see Table 1), transport of diphenylamine through the cuticle in solution in the solvent is thought to be unlikely. The degree to which a solvent induces rapid penetration of an insecticide through the insect cuticle will be referred to in future as its carrier efficiency.

Wigglesworth (1944) has shown that when the waxes covering the surface of the cuticle are removed by abrasion with hard dusts, evaporation of water from the underlying layers of the cuticle readily takes place and death of the insect from desiccation ensues. It is clear, therefore, that the wax must be in contact with a free-water surface which is continuous throughout the cuticle and has its source in the body fluids of the insect. Thus the structure of the insect cuticle, although highly complex, may be considered simply as a two-phase system comprising an outer phase of lipophilic elements associated with the epicuticle and an inner phase of hydrophilic elements associated with the exocuticle and endocuticle traversed by lipophilic elements extending from the hypodermis to the epicuticle (see Fig. 1). This conception of cuticle structure was adopted as a basis for the clearer understanding of the problem.

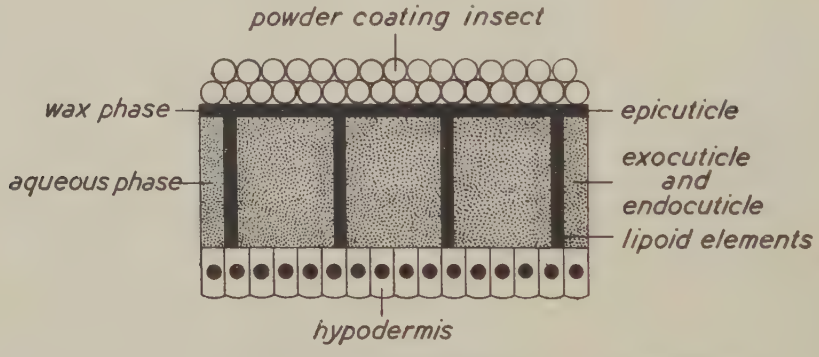

Fig. 1. Diagram showing the insect cuticle considered as a two-phase system.

PENETRATION OF THE WAX PHASE OF THE CUTICLE

The differences observed in carrier efficiency of the various solvents may be due to their ability to dissolve or penetrate the outer wax layer of the insect cuticle. As it has been shown by Beament (1945) that this wax is similar in composition to beeswax, the rate of penetration of the solvents through artificial beeswax membranes was taken as an approximate measure of their relative rates of penetration through the wax on the cuticle. A large number of these membranes was prepared in the following manner. Pieces of muslin supported by card impregnated with celluloid were dipped in a solution of beeswax in carbon tetrachloride. In this way a lamina of beeswax strengthened by threads of muslin and conforming to an approximate standard of thickness was obtained. A circular glass cell was then cemented on to the card surrounding the membrane and filled with a measured volume of solvent. The time taken for the solvent to pass through the beeswax at 30° C. was then observed. This was repeated twelve times for each solvent and the mean of the results obtained was taken as a measure of the rate of penetration of the solvent through beeswax. These figures are given in the first column of Table 2.

On comparing the rates of penetration of the solvents through beeswax with their observed carrier efficiency with diphenylamine (see Table 2), little correlation exists between them except that aniline, carbitol and castor oil, those solvents either penetrating slowly or not at all, agree in showing little or no carrier efficiency. Dimethyl-aniline, anisole, and *o*-dichlorobenzene, however, penetrate beeswax rapidly and also show no carrier efficiency. Although the ability of a solvent to penetrate the wax covering the insect cuticle appears to be essential for high carrier efficiency, it is evidently not the only factor involved.

The passage of wax solvent from the external surface of the insect to the hypodermis may take place via the lipophilic elements along which the wax secretions of the hypodermal cells pass to the epicuticle. Where diphenylamine is dissolved in the solvent, however, unless the solvent passes through the cuticle in bulk, it does not follow that the insecticide will be carried through the cuticle with it. It has been shown by Hurst (1943), and Läuger, Martin & Müller (1944), that insecticides whose molecules contain both an oil-soluble group and a water-soluble group, by orientating themselves at an oil/water interface, pass rapidly through insect cuticle by diffusion along such an interface. Although this mode of penetration may be open to those solvents possessing a molecule of this type, it is evident that it is a property inherent in the solvent alone and

not affect the passage through the cuticle of an ecticide lacking a water-soluble group, even ugh that insecticide is dissolved in the solvent n on the external surface of the cuticle. The rapid etration of diphenylamine in the presence of ols, xylenol and benzyl alcohol does not appear e due solely to its passage through lipophilic ele- nts since, if this were so, there seems no reason r dimethyl-aniline, anisole, and *o*-dichloro- zene, in view of their high rate of penetration ough beeswax, should not also increase the rate penetration of the insecticide. Transport of di- nylamine across the aqueous phase of the cuticle therefore, the next consideration.

of solution of the organic solvents and insecticides in the wax and aqueous phases of the insect cuticle than those taken after equilibrium between solute and solvent had been established. The solubility figures given in the tables cannot, therefore, be taken as physical constants.

The partition coefficients of the solvents between beeswax and water were then determined in the following manner. To 400 ml. of boiling distilled water was added 4 ml. of a mixture of equal parts by weight of molten beeswax and solvent. This was then shaken for 5 min., cooled to 30° C., and filtered to remove the solid beeswax. The volume of solvent leaving the beeswax and entering the water was then

Table 2. *Physical properties of solvents*

Solvent	Rate of penetration through beeswax at 30° C.	Solubility in water in c.c./100 c.c. at 30° C.	Partition coefficient between beeswax and water at 30° C.	Boiling point in ° C.
o-Cresol	1·5 min.	2·4	0·0093	191
m-Cresol	3·5 min.	2·1	0·0950	200
Xylenol	1·5 min.	0·4	0·0132	216–217
Benzyl alcohol	32·0 min.	4·1	0·0950	206
p-Cresol	12·0 min.	1·5	0·0033	201
Octyl alcohol	1·5 min.	0·1	0·0013	185
4-Methyl-cyclohexanol	4·0 min.	0·5	0·0021	175
Quinoline	12·0 min.	0·5	0·0200	238
Cyclohexanone	8·0 min.	7·1	0·015	155
Diacetone alcohol	45·0 min.	∞	High	164
Cyclohexanol	20·0 min.	3·5	0·0950	161
Acetophenone	20·0 min.	0·025	Nil	201
Benzonitrile	20·0 min.	0·1	Nil	190
Aniline	2 hr. 30 min.	3·6	0·0450	184
Carbitol	15 hr.	∞	High	198
Dimethyl-aniline	1·0 min.	Nil	Nil	193
Methyl benzoate	7·0 min.	0·15	0·0017	200
Castor oil	Nil	Nil	Nil	>200
Anisole	1·0 min.	0·1	0·0009	154
o-Dichlorobenzene	0·1 min.	Nil	Nil	180

PENETRATION OF THE AQUEOUS PHASE OF THE CUTICLE

The partition coefficient of solvents between beeswax and water

factor governing the passage of a solvent from the x phase of the cuticle into the underlying aqueous ase is the partition coefficient of the solvent be- en wax and the aqueous medium permeating the - and endo-cuticles. As the solubility of the vents in water is one factor governing their distri- ion between these two phases, this was deter- ned at 30° C. for each solvent. These solubilities ich are given in the second column of Table 2 and solubility figures appearing in succeeding tables re determined by a rough method in which the ute was shaken with the solvent for a short period ime only. It was considered that figures obtained his way would more nearly represent the degree

determined by measuring the amount of solvent still required to saturate the solution, and subtracting this figure from that already obtained for the solubility of the solvent in water at 30° C. The partition coefficient for each solvent was then expressed as the concentration of solvent in water over the concentration of solvent in beeswax. These figures are given in the third column of Table 2.

When a solvent, having penetrated the wax phase of the cuticle, passes into the aqueous phase beneath, it would be expected to increase the rate of penetration of diphenylamine if it either transported the insecticide across the aqueous phase, or rendered that phase more permeable to it. Any solvent shown to be unable to leave the wax phase would seem to be incapable of influencing the rate of penetration in either of these ways. In this connexion, therefore, it is significant that acetophenone, benzonitrile, dimethyl-aniline, castor oil, and *o*-dichlorobenzene,

those solvents in which the partition coefficient is nil, all show very low or negative carrier efficiency. The remaining solvents, with the exception of aniline, carbitol and castor oil which are unable to penetrate wax, all pass more or less readily from wax into water. These include, however, quinoline, cyclohexanone and cyclohexanol, which, in addition to a high partition coefficient, also have a high rate of penetration through beeswax, and would, therefore, be expected to possess a carrier efficiency as high as that of the cresols, xylenol and benzyl alcohol. Since the carrier efficiency of these two groups of solvents has been shown to differ considerably, it is evident that factors other than those already considered are involved.

The solubility of diphenylamine in solutions of solvents in water

The passage of a solvent from the wax phase of the cuticle into the aqueous phase would be unlikely to increase the permeability of that phase to diphenylamine, unless it tended to increase the rate of diffusion of the insecticide through the water permeating the cuticle. This might obtain in two ways. First, the continued passage of solvent from the wax phase into the relatively voluminous aqueous phase, by concentrating the solution of diphenylamine in solvent in the wax phase, would increase the percentage saturation of the insecticide in the aqueous phase in accordance with the partition coefficients of both diphenylamine and the solvent. Secondly, the presence of solvent dissolved in the aqueous phase might increase the solubility of diphenylamine in that phase. When a solvent is incapable of leaving the wax phase, diphenylamine will pass from the solvent into the aqueous phase at a rate governed by its percentage saturation in the solvent. This will decrease as the diphenylamine leaves the solvent and thus the rate at which the insecticide enters the aqueous phase will fall.

The solubility of diphenylamine in water and in solutions of the solvents in water at 80, 60, 40 and 20% saturation was measured at 30° C. In the case

Table 3. *Solubility of diphenylamine**

Solvent	Solubility in g./100 g. solvent at 30° C.	Solubility in mg./100 c.c. unsaturated solutions of solvent in water at 30° C.			
		80% sat.	60% sat.	40% sat.	20% sat.
o-Cresol	191	7·0	8·7	7·7†	7·5
m-Cresol	155	7·0†	7·3	8·5	5·8
Xylenol	137	9·2†	10·0	6·5	5·2
Benzyl alcohol	149	9·0†	10·0	7·5	6·0
p-Cresol	140	7·5	8·0	6·0	5·0†
Octyl alcohol	48	5·7	6·0†	5·0	5·0
4-Methyl-cyclohexanol	105	6·3	7·0	6·7†	5·0
Quinoline	270	3·0†	5·5	5·0	5·8
Cyclohexanone	355	3·5	2·8	2·0	2·0†
Diacetone alcohol	230	400·0	128·0	23·0	10·0†
Cyclohexanol	130	3·5	4·0†	3·5	3·0
Acetophenone	280	7·0	7·0	7·0	7·5
Benzonitrile	295	7·0	6·8	5·2	5·0
Aniline	295	3·5	4·5†	5·0	6·5
Carbitol	160	270·0	100·0	34·0	17·0†
Dimethyl-aniline	245	—	—	—	—
Methyl benzoate	225	2·0	2·0†	2·0	2·5
Castor oil	25	—	—	—	—
Anisole	265	4·0	4·0	6·5†	8·0
o-Dichlorobenzene	175	—	—	—	—

* Solubility of diphenylamine in water at 30° C. = 5·5 mg./100 c.c.
† Solubilities selected in Table 4.

of diacetone alcohol and carbitol, which are miscible with water in all proportions, a mixture of equal parts of solvent and water was chosen to represent a 100% saturated solution. The figures for these solubilities in solutions of the solvents used are given in Table 3.

Although, in the insect, it is impossible to estimate the percentage saturation of solvent in the water permeating the cuticle, an approach to the problem can be made by considering the factors governing it. As the water is in the substance of the cuticle and is free from convection currents, the concentration of solvent in water in the vicinity of the wax/water interface will tend to be relatively high. As the solvent diffuses away from the interface, however, the percentage saturation of solvent in the water will fall progressively as the hypodermis is approached. The degree of saturation of the aqueous phase with solvent will depend, first, on the partition coefficien

e solvent between wax and water; secondly, on lubility in water; and thirdly, on the quantity of nt available. It would, therefore, be expected a solvent with either a low partition coefficient high water solubility, and also a high volatility d give a solution in the aqueous phase at a lower ntage saturation than one with high partition icient or low water solubility, and with a lower ility. It is possible, therefore, to group the sol- according to the percentage saturation of the ous phase which might be expected to obtain in ving insect. This has been done in Table 4 and

Table 4

Solvent	Expected % saturation of solvent in aqueous phase	Solubility of diphenylamine in mg./100 c.c. of solution of solvent in water
noline	High	3·0
enol		9·2
resol		7·0
zyl alcohol		9·0
lohexanol	Medium	4·0
hyl benzoate		2·0
yl alcohol		6·0
ine		4·5
sole		6·5
ethyl-cyclo-exanol		6·7
resol		7·7
resol	Low	5·0
lohexanone		2·0
cetone alcohol		10·0
bitol		17·0
tophenone	Nil	Nil
zonitrile		Nil
ethyl-aniline		Nil
or oil		Nil
ichlorobenzene		Nil

re for the solubility of diphenylamine in a solu- of the solvent in water has been selected from ange given in Table 3. It is stressed, however, no accuracy is claimed for these saturation es, except that those solvents in the first group d give a higher percentage saturation than those e succeeding group and, likewise, with the d and third groups.

e high carrier efficiency of the cresols, xylenol benzyl alcohol compared with the lower carrier ency of quinoline, cyclohexanone and cyclo- ol has already been considered in the light of rates of penetration through beeswax and their ion coefficients, and it was decided that dif- ces in these two factors alone were insufficient count for the disparity shown. With regard to lubility of diphenylamine in solutions of these nts in water, however, it is seen that whereas the cresols, xylenol and benzyl alcohol increase the solubility of the insecticide in water, quinoline, cyclohexanone and cyclohexanol all depress its solubility. It is clear, therefore, that the solvents of the former group should induce a higher rate of diffusion of diphenylamine across the aqueous phase than those of the latter group.

Among the cresols, *p*-cresol shows a slightly lower carrier efficiency than its two isomers and this, too, may be correlated with a lower solubility of diphenylamine in solution of the solvent in water, though, in this case, the solvent also has a lower partition coefficient.

Methyl benzoate has been shown to have a negative carrier efficiency, yet this solvent penetrates beeswax more rapidly, and has a partition coefficient of the same order as *p*-cresol, where carrier efficiency is high. The very low solubility of diphenylamine in solutions of methyl benzoate in water may well explain this difference in carrier efficiency.

Thus, although diphenylamine may pass rapidly through the wax phase of the cuticle in the presence of certain solvents, which, in addition, may also be able to cross the wax/water interface, unless the presence of the solvent in the aqueous phase itself is favourable to solubility of the insecticide in that phase, high carrier efficiency will not be displayed by the solvent in question. A correlation is thus seen to exist between the high carrier efficiency of a solvent and those factors influencing diffusion of diphenylamine across the aqueous phase of the cuticle. Furthermore, it seems that this is achieved by a progressive increase in the percentage saturation of diphenylamine in both wax and aqueous phases, together with an increase in its solubility in the aqueous phase in the presence of the solvent.

It has already been suggested that high volatility of the solvent, by reducing the quantity of it available for passage into the aqueous phase, should play a part in determining carrier efficiency, and a further consideration of the importance of this factor is given in the next section.

THE EFFECT OF VOLATILITY OF THE SOLVENT

Any factor governing the percentage saturation of diphenylamine in the solvent in the wax phase of the cuticle will influence its distribution between the wax and aqueous phases, and will, therefore, affect its rate of diffusion across the aqueous phase. Evaporation of solvent from the powder coating the insect, although concentrating the solution of diphenylamine, will not only precipitate the insecticide after saturation is reached, thus reducing the total amount in solution, but will also reduce the volume of solvent available for solution in the aqueous phase. In these respects, high volatility of the solvent would not be expected to favour rapid diffusion of the insecticide across the aqueous phase. This is further compli-

cated by the solubility of diphenylamine in the solvents, the figures for which are given in Table 3. Where solubility for diphenylamine is high in relatively non-volatile solvents, the low initial percentage saturation of the insecticide cannot be increased by subsequent evaporation of the solvent, even though it may be increased by diffusion of the solvent into the aqueous phase. Such factors will limit the carrier efficiency of quinoline, acetophenone, benzonitrile, dimethyl-aniline, and methyl benzoate, all relatively non-volatile solvents in which the solubility of diphenylamine is very high. Neither will diffusion of the solvent into the aqueous phase be operative in these cases in improving the initial low percentage saturation of diphenylamine, except in the case of quinoline whose partition coefficient does not preclude this taking place. This agrees with the observed carrier efficiency of these solvents which has been shown to be either very low or nil.

The factors determining the carrier efficiency of a solvent so far discussed would seem to account for most of the differences in effect observed in the series of solvents tested. There are, however, two notable exceptions. There is a difference in carrier efficiency, first between *p*-cresol and cyclohexanol, and secondly, between benzyl alcohol and diacetone alcohol, which cannot be explained by any differences in their penetration rates through beeswax, partition coefficients, or solubility of diphenylamine in solutions of solvent in water. From a consideration of these factors alone, cyclohexanol and diacetone alcohol should possess a carrier efficiency approaching that of *p*-cresol and benzyl alcohol (see Table 2). From the list given in Table 2 it will be seen, however, that both cyclohexanol (161° C.) and diacetone alcohol (164° C.) have boiling points considerably lower than those of *p*-cresol (201° C.) and benzyl alcohol (206° C.). The higher volatility of the former solvents may well be the factor responsible for their relatively low carrier efficiency.

To verify the effect on carrier efficiency of the volatility of a solvent with a low boiling point, powders containing (*a*) 1·5% of cyclohexanol and (*b*) 1·5% of diacetone alcohol, each with and without 0·25% diphenylamine, were tested against keds, maintained in atmospheres saturated with solvent vapour. The initial percentage of solvent in the powder was increased in this instance to allow for evaporation during mixing. It was found that keds treated with cyclohexanol + insecticide died in 5 hr. and those treated with diacetone alcohol + insecticide in 3·5 hr., while keds dusted with the control powders died in 14 and 25 hr., respectively. These figures for solvent + insecticide show a reduction in the time of kill, over and above the times obtained in air, of 12 hr. in the case of cyclohexanol and 9·5 hr. for diacetone alcohol (see Table 1). It is evident from this experiment that the volatility of a solvent does play a major part in determining carrier efficiency where evaporation from the powder is rapid.

THE FACTORS GOVERNING SYNERGY BETWEEN DIPHENYLAMINE AND ORGANIC SOLVENTS

The influence of certain physical properties o organic solvents of diphenylamine on the rate o penetration of that insecticide through the insec cuticle has now been studied. Five physical proper ties of a solvent have been investigated in turn an their individual effects linked with observed carrie efficiency. The combined effect of these propertie when present to varying degrees in a single solvent however, has not yet been examined.

In Fig. 2 the relative value of the figures obtaine for the four major factors on which carrier efficienc of solvents has been shown to depend is expressed i diagrammatic form. The figures for each factor hav been grouped into four categories, namely, high medium, low and nil. These cover as far as possibl numerical groups. The key to the grouping for eac factor is appended. The solvents, likewise, are di vided into high, medium, low and nil. These value refer to their carrier efficiency as already given i Table 1 and, again, divisions have been made wher grouping of the figures most nearly occurs.

The fifth physical property shown to influenc carrier efficiency, namely, the solubility of diphenyl amine in the solvent, has been omitted from th diagram. In those solvents with active carrie efficiency the differences in solubility of the insecti cide, as shown in Table 3, are seen to be com paratively slight, with the exception of octyl alcohol the only case in which this factor may exert a specifi influence. Further reference to this solvent will b made below.

The diagram shows that all those solvents wit high or medium carrier efficiency exhibit either high or medium rating for each of the four factors with the exception of quinoline, cyclohexanone an diacetone alcohol, which occur at the end of th medium group. Those solvents in which carrie efficiency is low or nil, on the other hand, show a lo or negative rating for at least one factor in each case The three solvents with the highest carrier efficiency *o*-cresol, *m*-cresol and xylenol, are alone in pos sessing a high rating for all four factors. Thus, a hig degree of correlation exists between carrier efficienc of the solvents and their physical properties. Thi supports the view that the properties examined ar at least the principal factors involved.

As the carrier efficiency of a solvent represents th sum of effects of a number of distinct factors, it i essential to consider the importance of each in it relationship to the others, rather than as an entity t be treated separately. Thus, carbitol, and to a lesse degree aniline, through inability to penetrate th outer wax phase of the cuticle, show a low carrie efficiency in spite of high rating in all other factors Quinoline, on the other hand, which is deficient onl in reducing solubility of diphenylamine in water, ha

Carrier Efficiency	SOLVENT	A	B	C	D
HIGH	o-Cresol	●	●	●	●
	m-Cresol	●	●	●	●
	Xylenol	●	●	●	●
	Benzyl Alcohol	◍	●	●	●
	p-Cresol	●	◍	◍	●
MEDIUM	Octyl Alcohol	●	◍	◍	◍
	4-Methyl-cyclohexanol	●	◍	●	◍
	Quinoline	●	●	○	●
	Cyclohexanone	●	●	○	○
	Diacetone Alcohol	◍	●	●	○
LOW	Cyclohexanol	●	●	◍	○
	Acetophenone	●	Ⓝ	○	●
	Benzonitrile	●	Ⓝ	○	●
	Aniline	○	●	◍	◍
	Carbitol	○	●	●	●
NIL	Dimethyl-aniline	●	Ⓝ	Ⓝ	●
	Methyl Benzoate	●	◍	○	●
	Castor Oil	Ⓝ	Ⓝ	Ⓝ	●
	Anisole	●	○	●	○
	o-Dichlorobenzene	●	Ⓝ	Ⓝ	◍

	HIGH ●	MEDIUM ◍	LOW ○	NIL Ⓝ
Rate of penetration through beeswax	<30m.	30-60m.	>60m.	∞
Partition coefficient between wax and water	>0.005	0.005-0.001	<0.001	nil
Solubility of diphenylamine in solution of solvent in water	>6.0mg.	6.0-4.0mg.	<4.0mg.	nil
Boiling point (volatility)	>190°C.	190-170°C.	<170°C.	nil

Fig. 2.

a much higher carrier efficiency. Rapid penetration through wax, therefore, is of greater importance in determining the carrier efficiency of a solvent than high solubility of diphenylamine in a solution of that solvent in water.

Anisole and cyclohexanone agree in possessing a high rate of penetration through wax and a low boiling point, but differ in the remaining two factors, anisole having a low partition coefficient and a high solubility for diphenylamine in solution of the solvent in water, while for cyclohexanone the values for these two factors are reversed. In these two solvents, in which two of the factors have a high rating and two a low rating, it is evident that the lower carrier efficiency of anisole is determined by its low partition coefficient, which precludes its passage in any quantity from the wax phase to the aqueous phase where its presence would increase the solubility of diphenylamine in water. Other examples of the effect of negative partition coefficient in a solvent with high rate of penetration through wax are given by acetophenone, benzonitrile, dimethyl-aniline, and *o*-dichlorobenzene, in all of which carrier efficiency is also nil. Methyl benzoate is an example of a solvent in which negative carrier efficiency is due to the combined effect of a medium partition coefficient with low solubility of diphenylamine in solutions of the solvent in water.

High volatility of a solvent, as shown by a low boiling point, limits the effect of other factors. The carrier efficiency of diacetone alcohol and cyclohexanol is lower than would be anticipated from a consideration of these other physical properties, but, as will be remembered, these two solvents showed improved carrier efficiency when operating in atmospheres saturated with their vapour. Further, *p*-cresol and 4-methyl-cyclohexanol, apart from the fact that the latter increases the solubility of diphenylamine in water to a slightly greater extent, otherwise differ only in volatility. The greater volatility of 4-methyl-cyclohexanol is, likewise, considered to limit carrier efficiency.

The comparatively high carrier efficiency of octyl alcohol is not readily understood in the light of a medium rating for partition coefficient, solubility of diphenylamine in a solution of the solvent, and boiling point. In this instance, the low solubility of diphenylamine in the solvent, by providing an initial percentage saturation of the insecticide much higher than that obtaining with the other solvents, in which diphenylamine is far more soluble, may be responsible for the high carrier efficiency observed.

It thus appears that certain organic solvents, such as the cresols, benzyl alcohol, and xylenol, exert a high carrier efficiency with diphenylamine by greatly increasing its rate of diffusion across the insect cuticle. Diphenylamine alone, when applied to the cuticle as a solid, undoubtedly penetrates by diffusion both through lipoid elements and, to a limited extent, through the aqueous region. The presence of certain

solvents appears to favour its diffusion across the cuticle, first, by transporting the insecticide to the interface between the wax layer and the underlying aqueous layer (exo- and endo-cuticles); secondly, by concentrating it at the interface as the solvent is passing into the aqueous phase and thereby increasing its diffusion gradient across the interface; and thirdly, by increasing the solubility of the insecticide in the aqueous phase, and thus raising its partition coefficient between the wax phase and the aqueous phase.

The factors which determine in any solvent the efficiency with which this process is carried out are those influencing the percentage saturation of diphenylamine in the solvent in the wax phase, the rate at which penetration of the wax phase takes place, the partition coefficient of the solvent between the wax phase and the aqueous phase, and the extent to which the solvent increases the solubility of diphenylamine in the aqueous phase.

Results show, with three exceptions, a medium carrier efficiency for each of the mixtures tested. This represents a reduction in time of kill of keds of at least twelve hours over and above that of carbitol or aniline when used alone with diphenylamine (see Table 1). Mixtures of equal parts of two solvents, one of which tends to remain in the wax phase, would not be expected to show a carrier efficiency as high as a pure solvent, such as *o*-cresol. Whereas, with a pure solvent, passage from the wax phase to the aqueous phase results in the development of a high percentage saturation of diphenylamine at the interface, in the mixture, only half of the total volume can pass into the aqueous phase, the other half remaining in the wax and limiting the percentage saturation of diphenylamine at the interface. Furthermore, in mixtures of solvents, the physical properties of each constituent will tend to be modified and the carrier efficiency of the mixture will not be entirely predictable from data already obtained for the pure solvent. It is certain, however, that the highest value for each property in either constituent cannot be taken to represent that of the mixture.

Table 5. *Diphenylamine with mixed solvents*

Solvents	Time of death of keds in hr.		Rate of penetration through beeswax at 30° C.
	Mixed solvents + insecticide	Mixed solvents control	
Carbitol + acetophenone	20	—	1 hr. 45 min.
Carbitol + benzonitrile	> 30	—	1 hr. 15 min.
Carbitol + dimethyl-aniline	11	—	6 min.
Carbitol + methyl benzoate	9	28	9 min.
Carbitol + *o*-dichlorobenzene	11	—	5 min.
Aniline + acetophenone	10	*c.* 30	23 min.
Aniline + benzonitrile	28	—	> 24 hr.
Aniline + dimethyl-aniline	9	30	6 min.
Aniline + methyl benzoate	10	28	11 min.
Aniline + *o*-dichlorobenzene	12	—	2 min.

THE CARRIER EFFICIENCY OF MIXED SOLVENTS

Carbitol and aniline have been shown to possess all those physical properties necessary for high carrier efficiency with the exception of ability to penetrate the outer wax layer of the cuticle. Solvents, such as dimethyl-aniline and *o*-dichlorobenzene, on the other hand, show high rates of penetration through wax and are deficient in almost all the remaining factors. It should be possible, therefore, to employ mixtures of solvents which would lack none of the four factors and would display carrier efficiency higher than that of either of the constituents.

A series of powders was prepared employing 1 % of mixed solvent, comprising equal parts by volume of the two solvents tested, and 0·25 % diphenylamine, on china clay. These powders, together with a control series containing 1 % of mixed solvent alone were then tested against keds. The solvent mixtures used, the results obtained, and the rates of penetration of the mixtures through beeswax membranes are given in Table 5.

The addition of acetophenone and benzonitrile to carbitol, and of benzonitrile to aniline, produced little or no increase in carrier efficiency. This can be correlated with the relatively slow rate of penetration of these mixtures through beeswax (Table 5). In contrast, the mixture of aniline and acetophenone, in spite of the presence of the latter solvent, penetrated beeswax readily and, in consequence, agrees with the other mixtures in showing an improved carrier efficiency. Thus, although others of the factors, such as partition coefficient and solubility of diphenylamine in solution of solvent in water, may have become modified by reason of the mixing together of two solvents, it is apparent that, in the case of those three mixtures showing low or negative carrier efficiency, the major limiting factor is penetration through the wax of the epicuticle.

The results of this experiment thus support the hypothesis that the rate at which a solvent increases

ffusion of diphenylamine across the cuticle is de-ndent on its physical properties. The application a mixture of two solvents, themselves showing no rrier efficiency, but with complementary physical operties, could otherwise hardly be expected to crease the rate of diffusion of diphenylamine.

EXPERIMENTS WITH OTHER INSECTICIDES

as has been supposed, carrier efficiency is a dif-sion phenomenon then there appears to be no ason why it should not extend to other insecticides edominantly oil soluble and incapable of orienta-n at, and rapid diffusion along, an oil/water terface.

Apart from the synergy found to exist between gh boiling tar acids and derris, mentioned in the troduction to this paper, all the work so far de-ribed on the carrier efficiency of solvents has been rried out on a single insecticide, diphenylamine. o ascertain whether this synergy can be extended other chemically pure insecticides, dixanthogen, nitrostyrene dibromide, and rotenone were chosen r experiment. Solvents were then selected to cover e range of carrier efficiency, from high to nil, ob-rved with diphenylamine, and powders were pre-red as before with 1 % of solvent and 0·25 % of ecticide in china clay. Results of trials with these ainst keds, together with the solubility of the ecticide both in the solvents and in solutions of e solvents in water, are given in Tables 6, 7 and 8.

With all the solvents used, excepting methyl nzoate with ω-nitrostyrene dibromide, and 4-ethyl-cyclohexanol with rotenone, a carrier effici-cy similar to that observed with diphenylamine as obtained for each of the three insecticides. The me of death of keds in 7 and 6 hr. when treated th rotenone in carbitol and in methyl benzoate, spectively, agrees with a time of death of 6 hr. for ds treated with rotenone* alone and is, therefore, ken to indicate a negative carrier efficiency.

Although methyl benzoate displays no carrier ficiency with diphenylamine, dixanthogen or rote-ne, it shows a medium carrier efficiency with ω-ni-ostyrene dibromide. This may be attributed to the oportionately higher solubility of ω-nitrostyrene bromide in solutions of methyl benzoate in water.

With rotenone, 4-methyl-cyclohexanol displayed higher carrier efficiency than with diphenylamine. This may, perhaps, be explained by the low solubility of rotenone in this solvent. For, in this instance, the partition coefficient of the insecticide between the solvent in the wax phase, on the one hand, and the aqueous phase, on the other, would be expected to be considerably higher than that of diphenylamine. This would result in more rapid diffusion of the insecticide across the aqueous phase.

In spite of their varying rates of action in the absence of solvent, the times of kill obtained for all the insecticides tested with *o*-cresol, xylenol and benzyl alcohol are approximately the same. It is evident, therefore, that where insecticides are in solution in solvents it is the rate of penetration of the solvent through the insect cuticle that largely determines the rate of action.

It appears from this experiment that the synergy observed to exist between diphenylamine and certain organic solvents can be extended to other insecticides. It does not follow, however, that the carrier efficiency of a solvent is necessarily similar for all insecticides, since variations both in the solubility of insecticide in solvent and in its solubility in solutions of solvent in water are contributory factors.

DISCUSSION

The ability of a solvent to facilitate the passage of an insecticide across the insect cuticle has been shown to depend on its physical properties. Rapid penetration of the waxes covering the epicuticle enables a solvent to reach the interface between this layer and the underlying aqueous layer. Not all solvents of beeswax, however, increase the rate of diffusion of the insecticide across the cuticle; only those which possess a high partition coefficient between beeswax and water. This indicates that the main channels of diffusion of the insecticide in the presence of solvent are through the hydrophilic elements of the cuticle and not the lipophilic elements. Where a solvent passes readily from the wax phase into the aqueous phase, the concentration of the insecticide in the wax phase will tend to increase and will favour its rapid diffusion across the interface. Rate of diffusion is further increased where the solvent raises the solubility of the insecticide in the aqueous phase.

* It was shown by Webb (1945*a*) that ground derris ot dusted on to keds in which all the spiracles were aled penetrated the cuticle slowly at 30° C. but not at at 20° C. Derris resin extracted from the root and ound was also used in later experiments (Webb, 1945*b*), nen it was found that penetration of the cuticle was pid at 30° C. but very slow at 20° C. The difference in haviour of the ground root and the ground resin, at ° C., was attributed to the higher concentration of the xic agent present in the latter preparation. It seems at this suggestion may be incorrect, since a powder ntaining only 0·25 % of rotenone, and unable to pene-ate the spiracles, caused death of keds in as short a time 6 hr. at 30° C. In spite of the high concentration of derris resin in ground root, most of this is probably contained in cellular elements and is, therefore, unable to come into contact with the surface of the insect. If the available resin is composed solely of particles liberated from parenchyma cells of the root during the process of grinding, then, as these are carried freely by air currents, it would be expected that ground root would act rather as a respiratory than as a contact insecticide. Where ground resin or rotenone is used, every particle of the insecticide is free to come into contact with the cuticle and, in a powder containing 0·25 % rotenone, this may be far greater in amount than the free particles in a sample of ground derris root, although in the latter the concentration of resin may be very much higher.

Table 6. *Dixanthogen**

Solvent	Time of death in hr. of keds treated with solvent + insecticide	Solubility in g./100 g. solvent at 30° C.	Solubility in mg./100 c.c unsaturated solutions of solvent in water at 30° C.			
			80 % sat.	60 % sat.	40 % sat.	20 % sat.
o-Cresol	3·0	∞	0·25	0·75	0·5	0·25
Xylenol	2·2	∞	0·5	1·0	0·75	0·5
Benzyl alcohol	3·4	∞	0·75	1·0	1·0	0·75
4-Methyl-cyclohexanol	5·0	∞	0·25	0·5	0·4	0·25
Carbitol	30	∞	24·0	15·0	9·0	3·0
Methyl benzoate	27	∞	*c.* 0·1	*c.* 0·1	*c.* 0·1	*c.* 0·1

N.B. Keds dusted with 0·25 % dixanthogen remain active after 30 hr.

* Solubility of dixanthogen in water at 30° C. = 0·25 mg./100 c.c.

Table 7. *ω-Nitrostyrene dibromide**

Solvent	Time of death in hr. of keds treated with solvent + insecticide	Solubility in g./100 g. solvent at 30° C.	Solubility in mg./100 c.c. unsaturated solutions of solvent in water at 30° C.			
			80 % sat.	60 % sat.	40 % sat.	20 % sat.
o-Cresol	3·0	83	9	13	9	8
Xylenol	3·0	55	10	19	18	11
Benzyl alcohol	2·2	55	10	15	12	11
4-Methyl-cyclohexanol	5·0	18	9	10	8	11
Carbitol	> 28	64	200	80	35	20
Methyl benzoate	13	135	3·0	4·5	6·5	7·5

N.B. Keds dusted with 0·25 % ω-nitrostyrene dibromide die in approximately 30 hr.

* Solubility of ω-nitrostyrene dibromide in water at 30° C. = 10.0 mg./100 c.c.

Table 8. *Rotenone**

Solvent	Time of death in hr. of keds treated with solvent + insecticide	Solubility in g./100 g. solvent at 30° C.	Solubility in mg./100 c.c. unsaturated solutions of solvent in water at 30° C.			
			80 % sat.	60 % sat.	40 % sat.	20 % sat.
o-Cresol	2·0	48	0·2	0·6	0·5	0·4
Xylenol	2·0	62	0·75	1·0	1·0	0·75
Benzyl alcohol	2·0	21	1·5	2·5	1·25	0·5
4-Methyl-cyclohexanol	3·0	1·0	0·2	0·25	0·25	0·2
Carbitol	7·0	2·7	1·5	1·2	1·0	0·75
Methyl benzoate	6·0	18	< 0·1	< 0·1	< 0·1	< 0·1

N.B. Keds dusted with 0·25 % rotenone die in 6·0 hr.

* Solubility of rotenone in water at 30° C. = *c.* 0·1 mg./100 c.c.

Many solvents showing no carrier efficiency were und to have temporary anaesthetic effects on keds, hich may have been due to inhalation of the vapour to rapid penetration of the solvent alone through e cuticle. Hurst (1943) has shown that solvents ay penetrate the cuticle by means other than dif-sion, but it seems inevitable that some diffusion ust take place. Although this may be true for some re solvents, it is held here that penetration by secticides, such as diphenylamine, is governed imarily by diffusion phenomena. When both the lvent and the insecticide exert toxic effects, these ay readily be distinguished when the ked is used a test insect as the cessation of heart beat in this sect can conveniently be taken as an index of ath. Although the small quantities of solvent in-lved were, in some cases, sufficient to produce mobility, death, when it occurred, took place wly. Thus, with benzyl alcohol, for instance, there rapid immobility of the ked, but death does not cur until 21 hr. The addition of dixanthogen, how-er, with which alone keds are still active after 30 hr., uses cessation of heart beat in 3·4 hr. Although the icity of the solvent alone may reduce the resistance the insect to the insecticide to a small degree, it is t held that such a difference would be sufficiently ge to affect the general trend of the results.

In examining the effect of homologous series of ohols and fatty acids on blowfly larvae, Hurst 43) concluded that diffusion phenomena did not count for the variations in their rates of penetration ough the cuticle. He notes in the series of alcohols they are ascended from C1 to C8 that their physical operties change uniformly in the same direction as ain length increases. He found that the rate of netration of the alcohols through the cuticle in-ased as the series progressed from C1 to C5, ere maximum rate of penetration was observed, d then decreased from C5 to C8. It seems that s may well be explained as a diffusion pheno-enon, as it would be expected that only those ohols capable not only of penetrating the wax vering the epicuticle but also of leaving the wax d entering the underlying aqueous medium would netrate insect cuticle rapidly. This would most adily be achieved by the C5 alcohol where the rtition coefficient between wax and water ap-oaches unity. The behaviour of the fatty acids, on e other hand, does not conform to this arrange-ent and, here, Hurst's suggestion of a strong polar eraction of fatty acids with the protein com-nents of the cuticle provides an explanation.

The carrier efficiency shown by mixtures of two vents which alone have no carrier efficiency but ich possess complementary physical properties oports a theory based on diffusion rates and parti-n coefficients. It may also provide an explanation the effects observed by Hurst on blowfly larvae of xtures of ethyl alcohol and kerosene. The failure ethyl alcohol alone to penetrate the cuticle may be e to an inability to pass through the epicuticle. In kerosene/ethyl alcohol mixtures, the kerosene dissolves the outer wax layer of the epicuticle and allows the mixture to pass through to the exocuticle, where diffusion of alcohol through the water permeating the cuticle may then readily take place. When the larva is returned to pure ethyl alcohol, although the outer layers of the wax will have been destroyed, the lipophilic elements comprising the remainder of the epicuticle, together with any wax precipitated from the kerosene coating the insect at the time of immersion in pure ethyl alcohol, probably form a barrier to continued penetration.

Increase in rate of penetration through the insect cuticle of a contact insecticide in the presence of solvents such as the cresols, xylenol and benzyl alcohol, is perhaps more readily appreciated when those factors influencing penetration of a pure insecticide, such as rotenone, are considered.

With solid insecticides of low water solubility, penetration will be limited initially by the hardness of the waxes covering the epicuticle. Wigglesworth (1945) has shown that these waxes vary in hardness in different forms of insect and this may be a primary factor contributing to differences in susceptibility to contact insecticides. Rhodnius, which is normally completely resistant to rotenone, has a wax of about the same degree of hardness as beeswax with a thin "cement layer" on the outer surface. Wigglesworth (1944) has shown, however, that when the cuticle of *Rhodnius* nymphs is first rubbed with abrasive dusts, death follows the subsequent application of rotenone in under 24 hr. It is possible, therefore, that contact insecticides penetrate the cuticle principally in those regions where there is damage to the epicuticle and secretion of liquid wax for its repair is actively taking place. After penetrating the epicuticle the insecticide may pass by diffusion through the bulk of the lipophilic elements traversing the cuticle from the hypodermis and also, to a limited extent, through those elements permeated by water. Here, the pore canals, passing from the hypodermis to the base of the epicuticle, may facilitate diffusion.*

Where the insecticide possesses both a lipophilic group and a water soluble group in its molecule, Hurst (1943) has suggested that orientation of the molecule between lipophilic and hydrophilic elements at the interface takes place, and that a rapid penetration of the cuticle occurs by two dimensional diffusion along such an interface. With an insecticide not possessing a molecule of this type diffusion

* Since this paper went to Press it has been shown by Wigglesworth in *Rhodnius prolixus* (Hemiptera) and by one of us (J. E. W.) in *Eomenacanthus stramineus* (Mallophaga) that the pore canals do not end at the base of the epicuticle, but pass through the epicuticle as far as the outer wax layer. Thus after an insecticide has penetrated the wax layer and has entered the aqueous cytoplasmic contents of the pore canals its passage into the body should be facilitated by streaming of the protoplasm within the canals.

Further details of this work on the structure of insect cuticle will be published elsewhere.

through lipophilic and hydrophilic elements must take place independently and far more slowly.

Where an insect is dusted with pure insecticide a gradient of diffusion is set up across the cuticle governed by the quantity of insecticide in contact with the epicuticle. As an excess of dust must be applied to ensure a maximum area of contact with the epicuticle, a quantity of insecticide far higher than that necessary to kill the insect must be used. When, however, the insecticide is in solution in a suitable solvent in the powder, the solvent, by dissolving the hard outer wax layer and rendering the epicuticle relatively fluid, enables the insecticide to pass rapidly by diffusion to the interface between the epicuticle and the exocuticle. Here, a high concentration of insecticide is built up by the passage of the solvent into the water permeating the exocuticle. This continuous layer of insecticide at the interface represents an area of contact far in excess of that obtaining when the insecticide is present as solid particles on the surface of the cuticle. The presence of a high concentration of insecticide at the interface also results in a sharp diffusion gradient across the interface, and insecticide passes into the aqueous phase in quantities governed by its partition coefficient between the two phases. In addition, where the presence of solvent dissolved in the aqueous phase increases the solubility of the insecticide, then this partition coefficient rises and diffusion of the insecticide both across the interface and across the aqueous phase is, in consequence, more rapid. Wigglesworth (1941) has stressed the importance of partition coefficients in determining the rate at which an insecticide will leave an oily base and enter the tissues of an insect, and it is clear that this phenomenon will be of equal importance in determining the rate of penetration through the cuticle itself.

Apart from the effect of a solvent on the diffusion rate of the insecticide across the aqueous phase, diffusion through the lipophilic elements traversing the cuticle continues and may or may not be increased slightly by the presence of the solvent. The use of a solvent in an insecticidal dust thus enables a far higher proportion of the insecticide to reach the tissues of the insect than is possible when the solid insecticide is used alone, and the concentration of insecticide can, therefore, be reduced considerably without loss of efficiency.

SUMMARY

1. Using *Melophagus ovinus*, the sheep ked, as test insect, it was found that certain organic solvents of diphenylamine, such as the cresols, benzyl alcohol and 4-methyl-cyclohexanol, greatly increased the rate of action of this insecticide. Others, such as carbitol and methyl benzoate, gave little or no improvement in the time of kill. The degree to which a solvent induces rapid penetration of an insecticide is referred to as its 'carrier efficiency'.

2. The influence of the physical properties of the solvents on carrier efficiency was investigated. It was found that a high carrier efficiency could be correlated with a high rate of penetration through beeswax, a high partition coefficient of the solvent between beeswax and water and a high solubility of insecticide in a solution of the solvent in water. The volatility of the solvent and the solubility of insecticide in solvent were also contributory factors.

3. Mixtures of two solvents, each showing no carrier efficiency but together possessing all the essential physical properties, were tested and showed a carrier efficiency considerably higher than that of either constituent. This is taken as supporting evidence that carrier efficiency depends on certain physical properties of a solvent.

4. Using a range of solvents shown to exhibit various degrees of carrier efficiency with diphenylamine, comparable results were obtained with dixanthogen, ω-nitrostyrene dibromide and rotenone and showed that the synergy could be extended to other insecticides.

5. It is suggested that certain solvents increase the rate of penetration of contact insecticides through the insect cuticle:

(*a*) By transporting the insecticide through the lipoid elements of the epicuticle to the interface between this layer and the water permeating the exocuticle.

(*b*) By concentrating the insecticide at the interface between the epicuticle and the exocuticle, as the solvent passes into the exocuticle, and thus increasing the diffusion gradient of the insecticide across that interface.

(*c*) By increasing the solubility of the insecticide in the water permeating exo- and endo-cuticles and thus, by raising its partition coefficient between solvent in the epicuticle and water in the exocuticle, further increasing its rate of diffusion, not only across this interface, but also through exo- and endo-cuticles to the hypodermis.

We wish to express our thanks to Dr V. B. Wigglesworth and Mr J. W. L. Beament for their helpful criticism and for permission to quote from their unpublished work; also to Mr D. A. Lambie who determined the solubilities of the insecticides in the solvents.

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FACILITATION IN SEA ANEMONES

I. THE ACTION OF DRUGS

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(With Four Text-figures)

INTRODUCTION

a series of papers on the nerve net of the Actinozoa, ntin (1935*a*, *b*, *c*, *d*) showed that the decisive tor in the neuromuscular activity of these animals an extreme development of facilitation. This is n most readily in the transmission of excitation m the nerve net to certain muscles, in particular marginal sphincter of *Calliactis parasitica* and longitudinal mesenteric muscles of *Metridium le*. These muscles do not respond to a single trical stimulus applied to the nerve net, but a ond stimulus following soon after causes an mediate contraction. This means that a single mulus which has no visible effect leaves behind it effect which facilitates the transmission of a second ulse to the muscle. This facilitating effect of a mulus dies away gradually, and it is the interval ween stimuli, or the amount of facilitation, which ermines the size of the response. This contrasts rply with the mechanism in vertebrate skeletal scle, where the strength of the stimulus determines size of the response.

Analysis of the process of facilitation was begun Ross & Pantin (1940) in a study of the action of s and other substances on the response of mones to stimulation. They concluded that their ults could be explained best if it were assumed t two distinct processes exist in the transmission excitation from nerve to muscle in anemones: a process of facilitation or sensitization of the romuscular junction, and (2) a process of excita- n which is ineffective unless sensitization has ady taken place.

t is possible that either or both these processes chemical in origin. Cholinergic and adrenergic ems of transmission are known to occur in phyla er than the Chordata. Substances such as tylcholine, choline esterase and adrenaline are nd in animals from most phyla. The widespread sitivity of animals to acetylcholine, adrenaline and ted substances suggests that chemical systems of ctional transmission occur in most animals. It been known for many years that coelenterates are sensitive to drugs (Romanes, 1885). It seemed likely, therefore, that a study of the effects of drugs on the facilitated responses of sea anemones might throw some light on the mechanism of facilitation.

* 1851 Exhibition Scholar from Canada, 1937–40.

METHODS

The sea anemone, *Calliactis parasitica*, was used in the experiments. As in Pantin's (1935) work on this animal, the responses of the marginal sphincter muscle were recorded on a smoked drum by means of a light spring lever. The stimuli consisted of condenser discharges delivered through non-polarizable Ag/AgCl electrodes. A relay of the type described by Hall & Pantin (1937) enabled the condenser to be charged and discharged at a frequency controlled by a metronome in the circuit.

The effect of a drug on the anemone was observed in the following way. First a series of responses in natural sea water was recorded. Then a quantity of the drug being tested was introduced into the sea water and any immediate effects on the behaviour of the animal noted. Records of the response to stimulation were taken at frequent intervals thereafter so that any changes in the size and character of the response could be detected. Animals stimulated for corresponding periods in natural sea water can be regarded as controls. *Calliactis* can be stimulated once every 5 min. for 5–6 hr. before any decline in the size or speed of the response becomes apparent. This point has been kept in mind in analysing the results. In no experiment has the anemone been stimulated intensely enough to cause the augmented responses associated with the onset of fatigue (Ross & Pantin, 1940).

RESULTS

The drugs used in the experiments can be divided into four groups: (1) drugs which affect *cholinergic* nerve endings in the vertebrates; (2) drugs which affect *adrenergic* nerve endings in the vertebrates; (3) drugs which affect vertebrate nerve and muscle in various ways but which do not belong to groups (1) or (2); (4) substances which have been detected

in actinians and other marine invertebrates and which might have some physiological function in these animals.

There are certain features of the drugs' effects which should be mentioned before the action of individual drugs is described. It was soon discovered that drugs affect anemones only at relatively high concentrations, usually not below $1 : 10^4$ (compare acetylcholine exciting leech muscle at $1 : 5 \times 10^8$). Drugs also act more slowly on actinians than on other animals. Whereas a few seconds or at most a few minutes is usually long enough to produce the full effect on vertebrates, most drugs exert their maximum effect on *Calliactis* only after 1–2 hr. Long exposure to any drug eventually leads to a depression of the response, even when the drug causes enhanced responses at first. This general depression cannot be distinguished easily from specific depressant effects which some drugs seem to produce. In describing the results, only those drugs that have an early depressant effect, i.e. appearing within 2 hr. at concentrations of $1 : 10^4$, and whose action is wholly depressant, have been regarded as specific depressant agents.

One interesting feature of the action of drugs on sea anemones is the resistance shown by these animals to drugs which have toxic effects on other animals. In every case the anemones were returned to natural sea water after the experiments, which usually lasted for several hours. Not once did an experiment cause the death of an animal, and the effects of the treatment had always passed off within 24 hr.

Group 1

Drugs tested in this group were acetylcholine and a few of the many substances which act on cholinergic junctions in the vertebrates, viz. eserine, atropine, nicotine and curare. Since Dale & Loewi and their colleagues showed the role of acetylcholine as the transmitter of excitation at parasympathetic nerve endings and at the endings in voluntary muscle in the vertebrates, a number of tests have been carried out to see if this chemical mechanism also exists in the invertebrates. Among the effectors which respond to acetylcholine are the cephalopod stomach (Ungar, 1936), the cephalopod heart (Kruta, 1936), the longitudinal muscles of the leech (Minz, 1932), the earthworm gut (Wu, 1939) and the crustacean heart (Welsh, 1939). The most notable failure of acetylcholine in the invertebrates is in the leg muscles of *Carcinus* (Katz, 1936). Acetylcholine has been detected in extracts of cephalopod ganglia (Bacq, 1935), holothurian longitudinal muscles (Bacq, 1935) and crustacean heart (Welsh, 1939). Choline esterase, the agent which destroys acetylcholine in the vertebrates as soon as the muscle is excited (Loewi & Navratil, 1926), has been found in most invertebrate tissues with the exception of the sphincter of *Calliactis* (Bacq & Nachmansohn, 1937).

Acetylcholine and eserine. Acetylcholine bromide (B.D.H.) was tested on *Calliactis* in concentrations ranging from $1 : 10^6$ to $1 : 10^3$ in sea water. Fig. 1 A shows a typical result at a concentration of $1 : 1500$ at *p*H 6·5. It is evident that no significant change occurs in the size or form of the response. In a few experiments a slight increase in the size of the response was observed between 15 and 30 min. after the drug was introduced, but this effect was no larger than increases which sometimes occur in untreated animals. After very long exposure, 4 hr. or more at $1 : 10^4$, the response becomes weaker, but this is probably the general depressant effect mentioned above.

As Bacq & Nachmansohn (1937) have shown that *Calliactis* sphincter contains negligible quantities of choline esterase, the failure of acetylcholine cannot be attributed to its destruction by this enzyme in the animal. Moreover, when acetylcholine is applied with eserine to protect it from the enzyme, both drugs at concentrations of $1 : 10^4$, the records obtained are similar to those in Fig. 1 A, where the effect of acetylcholine alone is shown. Eserine alone is also ineffective.

Acetylcholine causes no direct response of any part of the anemone with the exception that a concentration of $1 : 10^4$ will dilate the mouth (Pantin & Pantin, 1943). However, many other substances do this, so it cannot be regarded as a specific acetylcholine effect.

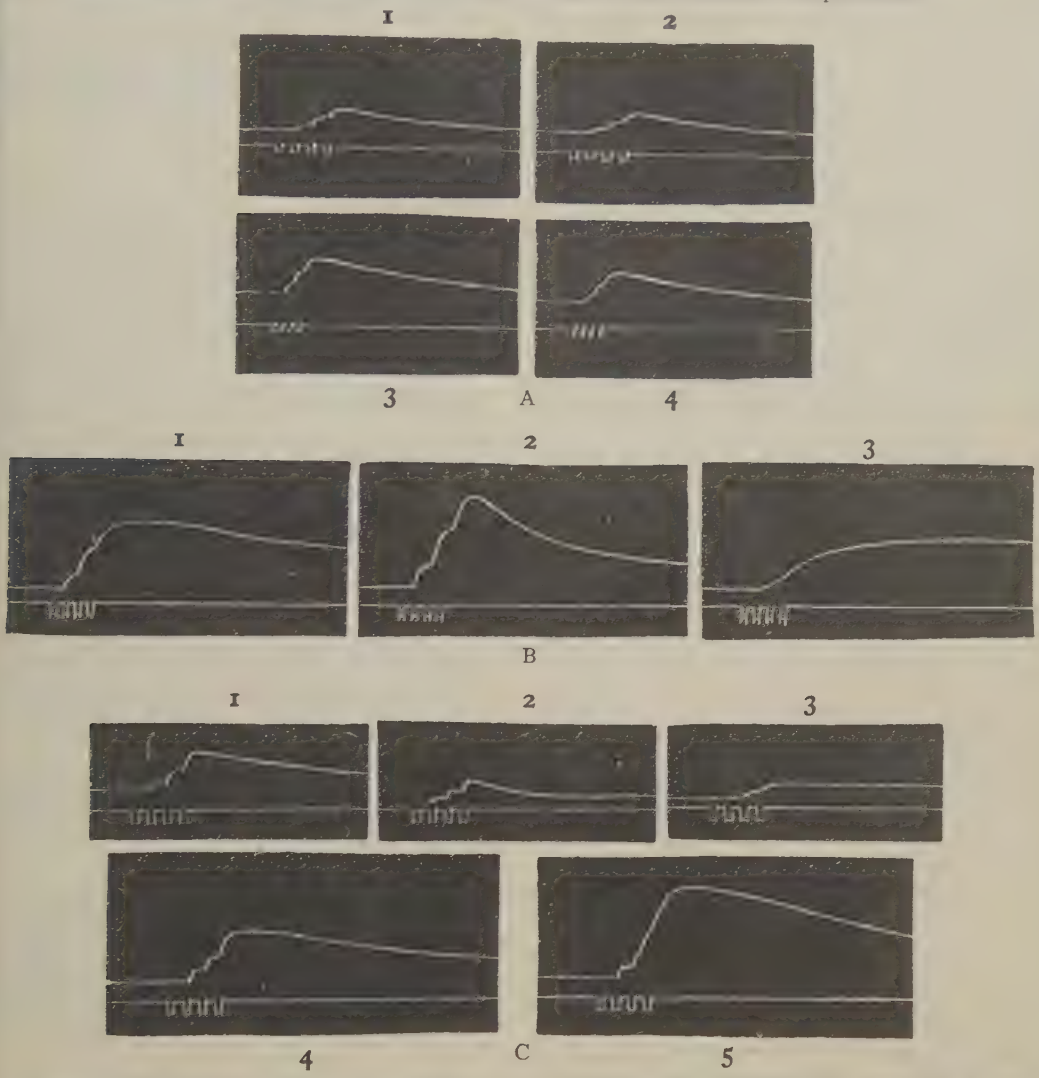
These experiments, therefore, give no evidence of any specific action by acetylcholine on *Calliactis*. This result and the absence of choline esterase in *Calliactis* strongly suggest that there can be no step in junctional transmission in anemones involving the liberation of acetylcholine at the nerve endings. Before deciding this point, however, the effects of some of the other drugs of the group should be considered.

Atropine. Atropine interrupts parasympathetic transmission in the vertebrates and abolishes the parasympathomimetic action of acetylcholine. It acts by preventing acetylcholine from reaching and exciting the effector cells. There are records of an action by atropine on certain coelenterates. Romanes (1885) found that it caused convulsive swimming movements in the jellyfish *Sarsia*. Moore (1917) was able to increase the rate of pulsation in *Gonionemus* by atropine. On *Metridium* he found that $1 : 2 \times 10^3$ atropine in sea water produced contractions of the tentacles and spasmodic longitudinal contractions of the whole animal.

Atropine enhances slightly the responses of *Calliactis* during the first hour at $1 : 10^4$ (*p*H 8·3). Later a gradual reduction in the size of the response sets in and after 3 hr. the steps in the staircase become indistinct and the whole response is very slow and prolonged. Both these effects can be seen in Fig. 1 B. At the same time the threshold of stimulation rises to about twice its original level in a typical experiment.

The action of atropine at 1 : 10^3 is qualitatively same, but the whole process is speeded up and depressant effect appears shortly after half an ır. Before this happens there is a period when ltiple responses or 'after-discharges' (Pantin,

response of *Calliactis* only occurs after 5 hr. exposure to atropine at 1 : 10^3. At lower concentrations the response is not abolished completely and the depression develops so gradually that it is difficult to regard it as a matter of importance.



1. Responses of *Calliactis* sphincter. A. Action of acetylcholine. Four stimuli at 1 in 1 sec. (1) in natural sea water; (2) after 155 min. in acetylcholine (1 : 1500). Four stimuli at 1 in 0·5 sec. (3) in natural sea water; (4) after 115 min. in acetylcholine (1 : 1500). B. Action of atropine. Four stimuli at 1 in 1 sec. (1) in natural sea water; (2) after 45 min. and (3) after 150 min. in atropine (1 : 10^4). C. Action of nicotine. Four stimuli at 1 in 1 sec. (1) in natural sea water; (2) after 180 min. and (3) after 250 min. in nicotine (1 : 10^4); (4) in natural sea water; (5) after 45 min. in nicotine (1 : 10^3).

5 *c*) tend to occur frequently. However, atropine s not cause spontaneous contractions of the incter or any other part of the animal.

lthough atropine has a depressant effect on *liactis*, this effect is hardly parallel to its action he vertebrates, where it abolishes the response ckly and completely. Complete abolition of the

Curare. In vertebrates curare blocks the passage of impulses across the neuromuscular junctions in voluntary muscle by preventing acetylcholine from reaching and exciting the muscle. It has no such effect on *Calliactis*. After 3–4 hr. exposure to curare (Merck) at 1 : 10^4, the response is unchanged except for the general depressant effect. It is interesting to

recall that Katz (1936) observed that curare has no effect at the neuromuscular junctions in the leg muscles of *Carcinus*.

Nicotine. Nicotine (B.D.H.) at a concentration of $1 : 10^4$ (*p*H 8·3) produces no effect on *Calliactis* for about 3 hr., but after that the response gradually diminishes in size. Larger doses ($1 : 10^3$) bring about a decline in the speed and size of the response after about 30 min. At this concentration, however, nicotine causes spontaneous contractions of the whole animal beginning about 5 min. after the drug is introduced and occurring frequently in the first half hour of the experiment. These contractions are usually maintained for some time, and it is often 5 min. before relaxation of the sphincter is complete. In the same period the responses to electrical stimuli frequently take the form of 'after-discharges' or multiple responses (Pantin, 1935*c*). In one experiment with nicotine at $1 : 10^3$, 'after-discharges' occurred in two out of three cases. Fig. 1 C shows the depressant action of nicotine at $1 : 10^4$ and an example of 'after-discharge' at $1 : 10^3$.

In vertebrates nicotine at first excites and then paralyses the autonomic ganglia and the nerve endings in voluntary muscle. These effects seem to resemble the effects observed on *Calliactis* where a period of spontaneous contractions is followed by a strong depressant action. However, tests on animals anaesthetized with magnesium show that the spontaneous contractions are due to impulses arising peripherally. When the anemone is immersed in a mixture containing equal portions of sea water and 0·4*M* $MgCl_2$, which according to Ross & Pantin (1940) anaesthetizes the sense organs in a few minutes, spontaneous contractions do not occur if the animal is then exposed to $1 : 10^3$ nicotine. It is therefore unlikely that this excitatory effect of nicotine on *Calliactis* is due to a specific action on the neuromuscular junctions as in the vertebrates.

Similarly, the depression of the facilitated response which occurs with nicotine appears to be of the general rather than the specific type. At $1 : 10^4$ its onset is so gradual that after 4 hr. the response is still one-half the normal size. It seems, therefore, that the similarity between the action of nicotine on vertebrates and on *Calliactis* is only superficial.

To sum up. Acetylcholine, eserine and curare fail to have any effects at all on *Calliactis*; atropine and nicotine fail to have effects that are truly analogous to those they cause at cholinergic junctions in the vertebrates. In view of these results and the absence of choline esterase in *Calliactis* spincter, it seems unlikely that the transmitting mechanism in anemones is the same as at those junctions where acetylcholine functions as the chemical transmitter.

Group 2

Included in the second group of drugs are adrenaline, epinine, ephedrine, tyramine, tryptamine, cocaine, ergotoxine and 933F. All these drugs act at sympathetic nerve endings in the vertebrates, and adrenaline, or some substance closely resembling adrenaline, is regarded as the chemical transmitter of the sympathetic system.

Few tests have been made on invertebrates with this class of drugs. Adrenaline has a depressant action on the holothurian cloaca (Wyman & Lutz, 1930), but it excites the cephalopod heart (Kruta, 1936) and stomach (Ungar, 1936). On the earthworm gut (Wu, 1939) adrenaline causes a contracture of the oesophagus, and strong doses ($1 : 10^5$) inhibit, while weaker doses ($1 : 10^7$) excite the 'crop and gizzard' preparation. Little work has been done on the occurrence of these substances in the invertebrates apart from the work of Henze (1929), who found large quantities of tyramine in the 'salivary glands' of cephalopods, and Bayer & Wense (1936), who reported that adrenaline can be detected in *Paramecium*.

Adrenaline. Adrenaline (adrenalina B.P., B.D.H.) was tested on *Calliactis* in concentrations varying from $1 : 10^6$ to $1 : 3 \times 10^3$ in sea water. Fig. 2 A shows a typical result at $1 : 10^4$. It is clear that adrenaline, the most active sympathomimetic drug in the vertebrates, has no effect on the anemone. Adrenaline is unstable in an alkaline medium, and the tests were carried out in acid sea water. Fig. 2 A shows a result at *p*H 5·4, and this accounts for the depression of the response that appears in the record. Effects of this kind are obtained when anemones are exposed to acid sea water for some time (Ross & Pantin, 1940).

Adrenaline, like acetylcholine, does not cause spontaneous contractions, 'after-discharges' or changes in excitability. It also has no effect on *Calliactis* when it is administered in conjunction with cocaine, which sensitizes tissues to the action of adrenaline in the vertebrates (Fröhlich & Loewi, 1910). Finally, the possibility that the failure of adrenaline is due to its destruction in the tissues has been ruled out almost entirely by tests (carried out by Dr H. Blaschko) which showed that *Calliactis* does not possess the amine oxidase that destroys adrenaline in various other animal tissues (Blaschko, Richter & Schlossmann, 1937).

Ephedrine. Ephedrine acts like adrenaline in the vertebrates, but because it is a more stable substance its effect is less transitory. Ephedrine hydrochloride (B.D.H.) was tested on *Calliactis* at concentrations of $1 : 5 \times 10^3$ and $1 : 10^3$. At the lower concentration there is no change in the response and no effect on behaviour or excitability. At $1 : 10^3$ ephedrine has an inhibiting effect after 2 hr. At first the response becomes progressively smaller, but after $3\frac{1}{2}$ hr. the steps of the staircase become obliterated and eventually several stimuli may be necessary to cause a response. This effect is so slow in developing that it must be regarded as an example of the general depressant action already mentioned. At any rate

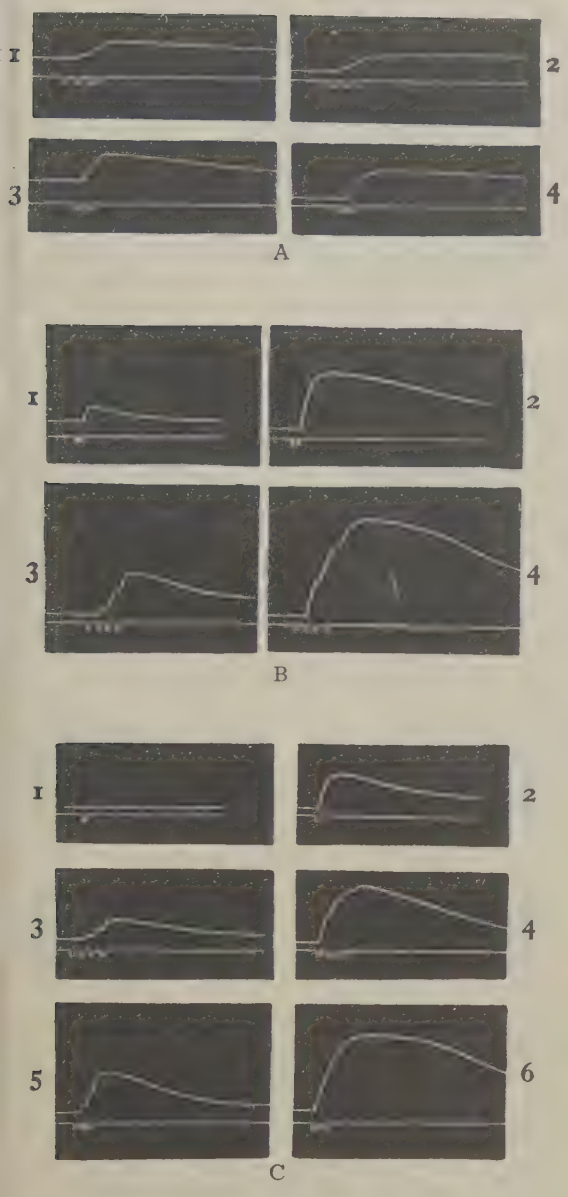


Fig. 2. Responses of *Calliactis* sphincter. A. Action of adrenaline. Four stimuli at 1 in 1 sec. (1) in natural sea water; (2) after 120 min. in adrenaline ($1 : 10^4$; *p*H 5·4). Four stimuli at 1 in 0·5 sec. (3) in natural sea water; (4) after 120 min. in adrenaline ($1 : 10^4$; *p*H 5·4). B. Increase in size of response with tyramine. Two stimuli at 1 in 0·5 sec. (1) in natural sea water; (2) after 87 min. in tyramine ($1 : 10^4$). Four stimuli at 1 in 1 sec. (3) in natural sea water; (4) after 84 min. in tyramine ($1 : 10^4$). C. Responses to single stimuli with tyramine. One stimulus (1) in natural sea water; (2) after 50 min. in tyramine ($1 : 10^4$). Four stimuli at 1 in 1 sec. (3) in natural sea water; (4) after 43 min. in tyramine ($1 : 10^4$). Four stimuli at 1 in 0·5 sec. (5) in natural sea water; (6) after 115 min. in tyramine ($1 : 10^4$).

it is clear that ephedrine has no effects on *Calliactis* analogous to its sympathomimetic effects in the vertebrates.

Epinine. Epinine (hydrochloride, Burroughs Wellcome) is even less effective than ephedrine on *Calliactis*. It has no effect at all at $1 : 10^4$ in sea water, and only very slightly depressant effects at $1 : 10^3$ (*p*H 8·3).

Tyramine. Unlike the three drugs of the group whose effects have just been described, tyramine proved to have important effects on the anemone. It was tested not only because it is another of the substances with an action like adrenaline in the vertebrates but also because it is a substance of some importance in marine invertebrates. Like adrenaline, it excites the heart (Kruta, 1936) and stomach (Ungar, 1936) of cephalopods. Sereni (1930) has shown that tyramine causes expansion of the chromatophores of cephalopods. As tyramine is produced in the 'salivary glands' of these animals (Henze, 1929) and removal of these glands causes permanent expansion of the chromatophores, Sereni (1930) put forward the view that tyramine exerts a humoral control over the chromatophores.

On *Calliactis*, tyramine hydrochloride (B.D.H.) first enhances considerably the size of the response. This effect develops rapidly. Fig. 2 B shows records taken 84 and 87 min. after the anemone was exposed to tyramine ($1 : 10^4$). The size of the enhancement is of the order of 3–4 times the size of the original response in natural sea water, and at this concentration it usually lasts for several hours.

In addition to enhancing the responses, tyramine may permit responses to single stimuli at any time after 30 min., thus altering the most distinctive feature of the facilitated response in *Calliactis*. Three examples of this effect are shown in Fig. 2 C occurring between 40 min. and 2 hr. In these records there are responses to the second, third and fourth stimuli as well as the first, but the first response is bigger than the succeeding ones.

At first the single-shock responses caused by tyramine appear very erratically. Before 2 hr. they occur in about 1 in 10 responses to stimuli. Between 2 and 3 hr., however, there is a period of about 30 min. duration when the effect occurs much more frequently. Afterwards the effect disappears completely, but not because the tyramine has been used up, as it is still effective on another animal. Eventually after 6 hr. exposure at $1 : 10^4$, and earlier at higher concentrations, the response deteriorates as the effects of fatigue and the general depressant action begin to operate.

In an effort to overcome the fortuitous character of the responses to single stimuli, cocaine was used in conjunction with tyramine in view of the sensitizing action of cocaine used in conjunction with adrenaline in the vertebrates (Fröhlich & Loewi, 1910). When the two drugs were applied together at concentrations of $1 : 10^4$ more consistent results

were obtained. In a typical test lasting 4 hr. two out of five responses occurred on the first stimulus, compared with about 1 in 10 with tyramine alone. This interaction of cocaine and tyramine on *Calliactis* recalls the sensitizing effect of cocaine used with adrenaline in the vertebrates.

Tyramine has no other effects on *Calliactis*. It does not alter the threshold of stimulation; it does not cause 'after-discharges'; it does not affect the behaviour of the animal, and spontaneous contractions have not been observed. This absence of the effects associated with a peripheral action suggests that the effect of tyramine on the size and character of the response is due to an action on the neuromuscular junctions themselves where the facilitation mechanism is located (Pantin, 1935*a*).

The effectiveness of tyramine suggests that the mechanism of transmission in *Calliactis* may be related in some way to the mechanism operating at adrenergic junctions in the vertebrates. However, it must be pointed out that the action of tyramine on the anemone is very different from the action of tyramine or adrenaline in the vertebrates. In the latter case these drugs are able to cause direct responses of the effectors with sympathetic innervation without nervous stimulation at all. In *Calliactis* there is no direct response to tyramine, and the effect of the drug is only revealed by certain changes that occur in the response to stimulation. Probably we are dealing here with a fundamental difference between these two neuromuscular systems which is reflected in the totally different character of the drug effects.

Tryptamine. Tryptamine or beta-indolethylamine (hydrochloride, Roche) has effects on *Calliactis* similar to those caused by tyramine. It increases the size of the response to stimulation and later permits responses to occur to single stimuli. Tryptamine is considerably less potent than tyramine in causing both these effects, and as with tyramine, no other change accompanies these effects on the response. There are no spontaneous contractions, no 'after-discharges' and no changes in excitability. Therefore, the tryptamine effect also suggests that a mechanism related to the mechanism of transmission at adrenergic junctions exists in anemones. But we have to note the fact that these effects are limited to changes in the response to stimulation and that direct effects of the type caused by tryptamine in the vertebrates do not occur in *Calliactis*.

Oxytyramine, tyrosine, hordenine and dorpan. Four substances which closely resemble tyramine in chemical composition, oxytyramine (hydrochloride, Merck), tyrosine (B.D.H.), hordenine (sulphate, Merck) and dorpan (Roche) were tested on *Calliactis* at concentrations between $1 : 10^4$ and $1 : 10^3$. None of them increased the size of the responses or caused responses to single stimuli. Except for slight depressant effects by tyrosine and oxytyramine they were completely ineffective.

Isoamylamine, ethylamine and oxyethylamine. The effects of a number of simpler amines on *Calliactis* were examined. Isoamylamine (hydrochlorate, Roche) had effects like those of tyramine, doubling the size of the response after 90 min. at a concentration of $1 : 5 \times 10^3$ and causing occasional responses to single stimuli. Ethylamine (hydrochloride, B.D.H.) had no effect at $1 : 10^4$ and had a depressant effect after 45 min. at $1 : 10^3$. Oxyethylamine or colamine (hydrochloride, Roche) was completely ineffective on *Calliactis* at concentrations of $1 : 10^4$ and $1 : 10^3$.

Cocaine. In vertebrates cocaine sensitizes tissues to the action of adrenaline and potentiates the response to sympathetic stimulation. It has been seen already that cocaine shows up the response to tyramine in a manner resembling its sensitizing effect in the vertebrates. Cocaine also enhances the response of *Calliactis* to stimulation. Fig. 3 A shows contractions which are about twice the normal size after 90 min. in cocaine at $1 : 10^4$. This result is similar to the augmentory action of cocaine on the nictitating membrane of the cat (Rosenblueth & Rioch, 1933). It must be noted, however, that cocaine does not cause responses to single stimuli

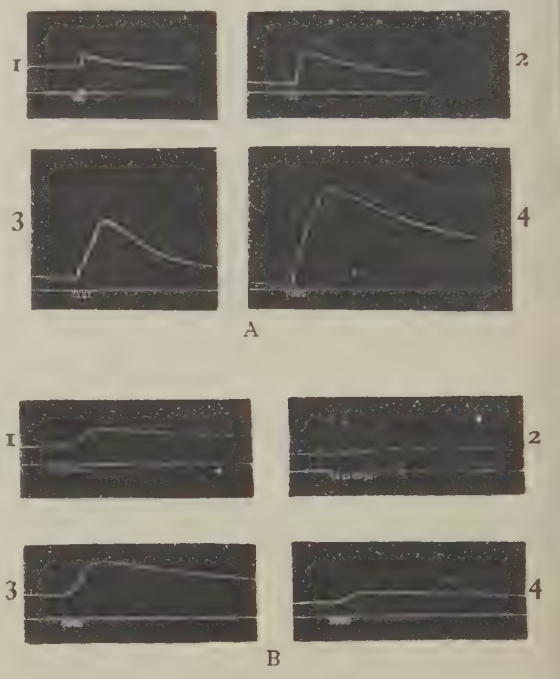


Fig. 3. Responses of *Calliactis* sphincter. A. Action of cocaine. Two stimuli at 1 in 0·5 sec. (1) in natural sea water; (2) after 90 min. in cocaine ($1 : 10^4$). Four stimuli at 1 in 0·5 sec. (3) in natural sea water; (4) after 90 min. in cocaine ($1 : 10^4$). B. Action of ergotoxine. Four stimuli at 1 in 1 sec. (1) in natural sea water; (2) after 150 min. in ergotoxine ($1 : 10^5$). Four stimuli at 1 in 0·5 sec. (3) in natural sea water; (4) after 150 min. in ergotoxine ($1 : 10^5$).

and that it has no effect on the behaviour of the anemone. It is particularly interesting that the well-known local anaesthetic action of cocaine does not appear in tests on *Calliactis*.

Ergotoxine. Ergotoxine abolishes the response of vertebrate smooth muscles to adrenaline and to stimulation through the sympathetic system. It also acts on certain invertebrates. Bacq (1932) reported that ergotamine paralyses the peripheral mechanism of the chromatophores of cephalopods and also suppresses the adrenaline effect on these structures. Wu (1939) found that ergotoxine antagonizes the action of adrenaline on the earthworm gut, but it has no effect in the absence of adrenaline.

Ergotoxine reduces considerably the size of the response of *Calliactis* to stimulation. Fig. 3 B shows the effect of ergotoxine (ethanesulphonate, B.D.H.) at $1 : 10^5$ after $2\frac{1}{2}$ hr. and the reduction of the response to approximately one-quarter its initial size. This depressant action recalls the action of ergotoxine in the vertebrates, although the complete suppression of the response was never observed. It is unlikely that this ergotoxine effect is an example of the general depressant action observed with most drugs after several hours, because ergotoxine was applied here in a relatively dilute solution, the drug not being available in a more soluble form.

Tests were also made to see if there is any interaction between ergotoxine and tyramine on *Calliactis*. When the anemone is exposed to ergotoxine ($1 : 10^5$) for 3 hr. and then placed in sea water containing tyramine at $1 : 10^4$, responses to single stimuli do not occur, and the enhancement of the response that occurs is slight compared to the big increases recorded with tyramine alone. Thus there is an interaction between ergotoxine and tyramine in *Calliactis* that resembles the interaction between ergotoxine and adrenaline in other animals.

Although ergotoxine reduces the size of the response in *Calliactis*, the second stimulus of a series continues to be the first effective stimulus. Responses delayed beyond the second stimulus were not observed. In this respect the ergotoxine effect resembles the depressant effects of carbon dioxide and magnesium on the neuromuscular junctions in *Calliactis* (Ross & Pantin, 1940).

933F. Bacq & Fredericq (1934) showed that the nictitating membrane of the cat ceased to respond to adrenaline after 933 F (piperidinomethylbenzodioxane), although the response to stimulation of the post-ganglionic fibres remained unaffected. This effect raised difficulties for the simple theory of chemical transmission at sympathetic junctions and was widely discussed (Monnier, 1936; Cannon & Rosenblueth, 1937).

933 F has important effects on *Calliactis*. For the first hour at $1 : 10^4$ (*p*H 8·3), the response and behaviour of the anemone remain unchanged, but after 2 hr. a steady increase in the size of the response becomes apparent. By 3 hr. this enhancement is very great (Fig. 4 A), exceeding that caused by calcium and potassium (Ross & Pantin, 1940) and bigger even than the tyramine enhancement. In Fig. 4 A the height of a single response on the smoked record at an interval of 1 sec. is increased almost ten-fold and approaches the limit of contraction of the muscle.

Between 3 and 4 hr. at $1 : 10^4$ another effect appears. As with tyramine, responses to single stimuli occur, at first occasionally, but between 4 and 5 hr. almost without fail. Fig. 4 B shows two examples of this effect. The size of these responses to single stimuli varies considerably. At times they almost amount to a complete closure of the sphincter, and in most cases they are bigger than the responses to single stimuli caused by tyramine. However, frequent stimulation seems to cause a falling off in the size of these responses and sometimes leads to a temporary return to the normal condition with responses occurring on the second stimulus.

After 5 hr. the responses to single stimuli disappear and the size of the response begins to decline. Eventually the depressant effects associated with fatigue and long exposure set in (6–8 hr.).

Following a response to a single stimulus with 933 F a period of inexcitability occurs. This effect, which did not occur with tyramine, is shown in Fig. 4 B (2) where all stimuli after the first fail to cause a response. The duration of this inexcitable period varies from one experiment to another, but it is usually about 15–20 sec. before even a slight response can be obtained. Fig. 4 B (3) shows a state of partial recovery after an interval of about 40 sec. No explanation of this effect can be advanced, but it would seem that, in causing responses to single stimuli, 933 F in some way exhausts the response mechanism temporarily.

These effects on the response are the only 933 F effects observed on *Calliactis*. Spontaneous contractions and 'after-discharges' do not occur more often than usual even when the effect on the response is at its maximum, and the behaviour of the anemone remains normal throughout the tests. Thus 933 F, like the other effective drugs discovered, exerts its effect on the response to stimulation, without causing responses itself. It should be pointed out that in the case of 933 F there is no similarity between the nature of its effect on *Calliactis* and on vertebrate tissues which respond to it.

The effectiveness of a few drugs of this group immediately suggests that a process, related in some respects to the chemical process at adrenergic nerve-endings, exists in *Calliactis*. The ineffectiveness of adrenaline and other substances shows that there are certain differences in the chemical nature of these processes. But it is important to note that although certain sympathomimetic substances are effective, they do not by themselves cause responses as in the vertebrates. Therefore, it is likely that a different type of hypothesis from the chemical theory of

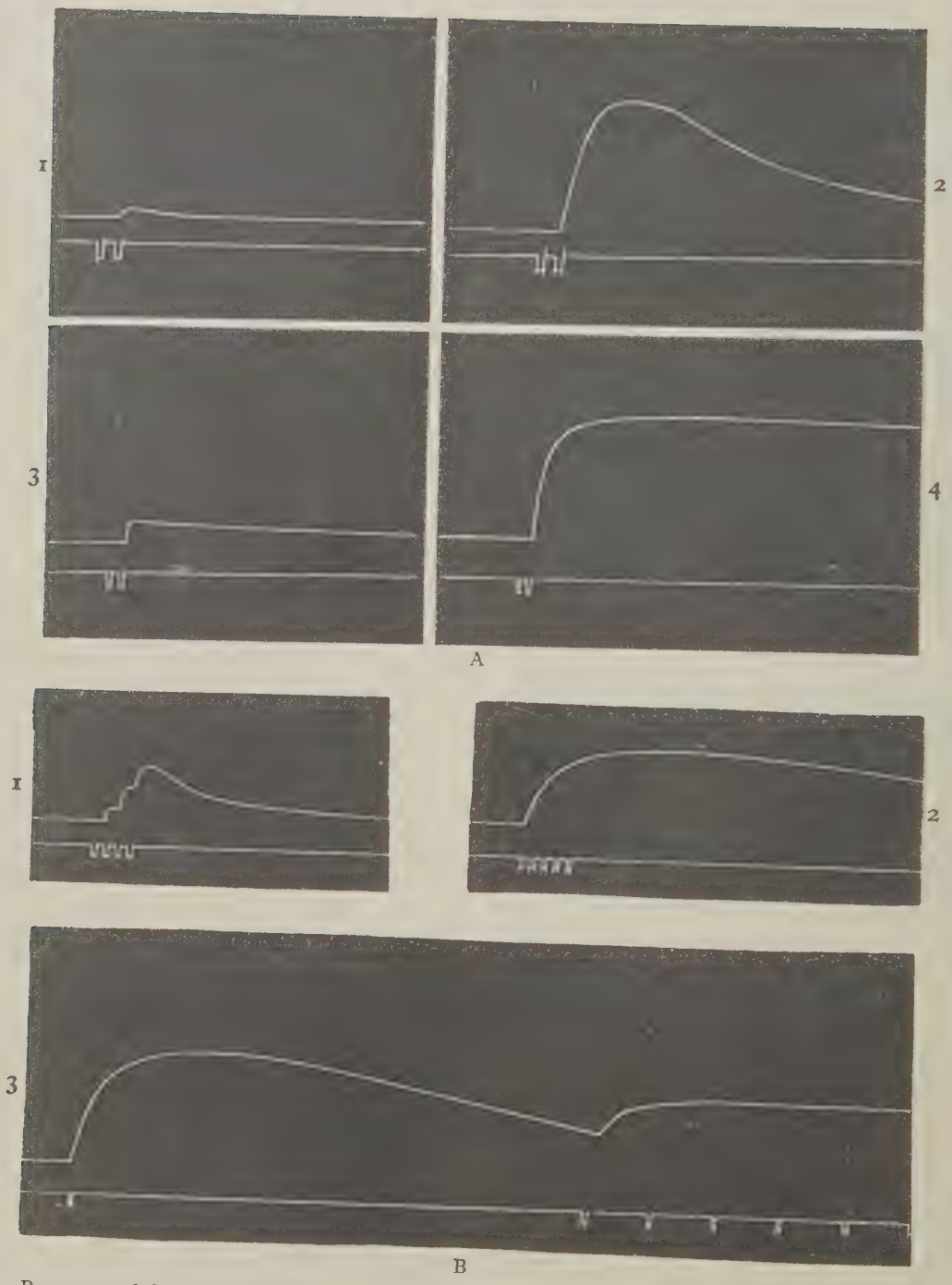


Fig. 4. Responses of *Calliactis* sphincter. A. Increase in size of response with 933 F. Two stimuli at 1 in 1 sec. (1) in natural sea water; (2) after 210 min. in 933 F (1 : 10^4). Two stimuli at 1 in 0·5 sec. (3) in natural sea water; (4) after 180 min. in 933 F (1 : 10^4). B. Responses to single stimuli with 933 F. (1) four stimuli at 1 in 1 sec. in natural sea water; (2) five stimuli at 1 in 1 sec. after 240 min. in 933 F (1 : 10^4); (3) one stimulus after 270 min. in 933 F (1 : 10^4) followed by second stimulus 40 sec. later and successive stimuli at intervals of 5 sec.

transmission will have to be advanced to explain these effects.

Group 3

Certain other drugs which affect vertebrate nerve and muscle, but are not specifically associated with cholinergic and adrenergic mechanisms of transmission, were tested on *Calliactis*, viz. strychnine, histamine, pituitrin, guanidine and veratrine.

Strychnine. In the vertebrates strychnine sensitizes the reflex centres of the central nervous system and exaggerates the normal reflex response. Romanes (1885) found that strychnine stops the pulsations of the jellyfish *Cyanea* and brings about a tonic contraction of the muscles. Moore (1917) reported that strychnine has no effect on *Aurelia* but it excites starfish, shrimps and cephalopods. He concluded from this that strychnine only affects the more highly organized nervous systems.

Nothing resembling the excitant effect on vertebrates was observed in tests with strychnine on *Calliactis*. It did not cause spontaneous contractions and at $1 : 10^4$ it had no effect on the response. At $1 : 10^3$ the response diminished gradually and after 3 hr., when the size of the response had been greatly reduced, several stimuli were necessary to cause a response. Like similar effects with magnesium this is probably due to the breakdown of through conduction in the nerve net (Ross & Pantin, 1940).

Histamine. Histamine causes most vertebrate smooth muscles to contract, and this is attributed to a direct action on the muscle cells rather than to junctional effects. Histamine has no such effect on *Calliactis*. At $1 : 10^4$ the response remains unchanged. At $1 : 10^3$ the response is delayed beyond the second stimulus after $2\frac{1}{2}$ hr., and as this is accompanied by a big increase in the threshold the effect is probably due to inexcitability.

Pituitrin. Pituitrin, which like histamine is a general stimulant of vertebrate smooth muscles, is completely ineffective on *Calliactis*. The tests were carried out with 0·5 and 2·5 c.c. of pituitary extract (Parke Davis), i.e. 5 and 25 i.u., in 500 c.c. of sea water.

Guanidine. Guanidine has an excitant effect on many vertebrate smooth muscles, especially on the wall of the alimentary tract. Sereni (1928) reported that it has a stimulating action when applied to the peripheral nerves of cephalopods. Moreover, guanidine derivatives are well known constituents of tissues from lower invertebrates including the Actinozoa (Kutscher & Ackermann, 1933). Guanidine (nitrate, B.D.H.) was tested on *Calliactis* at concentrations of $1 : 10^4$ and $1 : 10^3$. Only general depressant effects were observed.

Veratrine. Veratrine causes a characteristic contracture of vertebrate skeletal muscle after stimulation. On *Calliactis*, veratrine causes a marked reduction in the speed and size of the contractions. After $2\frac{1}{2}$ hr. at $1 : 10^4$, the response to a pair of stimuli is only one-tenth its original size. This is too marked a decline to be a general effect.

The anemone shows a more marked direct response to veratrine than to any other drug tested. At $1 : 10^3$, spontaneous contractions occur frequently. However, there is evidence that this is due to a peripheral action rather than to a direct action on the muscles. When veratrine was tested on animals that had been exposed to magnesium to anaesthetize the sense organs, spontaneous contractions did not occur.

In general, these five drugs have not had effects that resemble their effects on vertebrates. The failure of general stimulants of vertebrate smooth muscle like histamine, pituitrin and guanidine is important in showing the difference between the general pharmacological properties of the two systems.

Group 4

Tests were carried out on a few substances which have been detected in anemones or other marine invertebrates. Some of the drugs in the other groups could also be placed in this category, viz. tyramine, histamine and guanidine. Three other substances found in marine invertebrates were tested on *Calliactis*: tetramethylammonium hydroxide, which has been extracted from *Actinia equina* and is believed to be the poison contained in the nematocysts (Ackermann, Holtz & Reinwein, 1923); trimethylamine oxide, which is found in the muscles of many marine animals (Kutscher & Ackermann, 1933); betaine hydrochloride, which is related to γ-butyrobetaine detected in *Actinia equina* (Ackermann, 1927). The only effect obtained from these substances was a marked depressant action by trimethylamine oxide. After 90 min. at $1 : 10^3$ this substance abolishes the response altogether. No other drug is so effective as a depressant agent.

CONCLUSION

The experiments described above have shown that the neuromuscular system of the sea anemone *Calliactis parasitica* is sensitive to certain drugs which have sympathomimetic effects on the vertebrates. There is no evidence of any action by drugs of the parasympathomimetic group such as acetylcholine.

One feature of the results has been the absence of any direct effects by the drugs. Out of more than twenty-five drugs tested, only nicotine and veratrine caused spontaneous contractions, and these appeared to be due to a peripheral and not a muscular action. Moreover, the drugs whose effects on the response are most marked, tyramine, tryptamine and 933F, are singularly without effect in causing direct contractions of the animals. It is possible, however, that this absence of direct effects is related to the conditions of the experiments. We are working here with tissues that do not possess a vascular system as in the vertebrates, and drugs cannot be introduced

suddenly and at high concentrations at the particular spot where the effect is to be observed. The slow development of the responses to single stimuli caused by the effective drugs may be significant in this connexion. It is necessary, therefore, to bear in mind the limitations of the experiments and the delayed action of the drugs, although it seems unlikely that transmission by a single chemical step can occur in anemones in view of the absence of direct effects by the drugs.

The action of the effective drugs on *Calliactis* is different from that on vertebrates. Instead of direct excitation of the muscles by the drugs, the effects observed are confined to enhancement and sensitization. The latter effect takes the form of responses to single stimuli to which the anemone does not respond under normal conditions.

As a result of the action of ions on sea anemones, Ross & Pantin (1940) suggested that there are two processes in neuromuscular transmission: (1) a process of sensitization or facilitation which must precede (2) a process of excitation of the muscle. There was evidence that the first of these processes was chemical in nature, and the hypothesis was advanced that a 'facilitator' liberated at the nerve endings carried out the sensitization process. Some, but not all, the sensitization phenomena observed with certain drugs can be explained on this hypothesis.

On the 'facilitator' hypothesis three effects would be expected when an anemone is exposed to a substance with sensitizing properties: (1) enhancement of the response, (2) responses to single stimuli, (3) delayed decay of facilitation. The enhancing properties of the drugs tyramine, tryptamine and 933 F have already been noted and clearly agree with the expected result. It is a fact also that the same drugs cause responses to single stimuli, thus fulfilling the essential requirement of a sensitizing drug. However, there is a fortuitous element in this effect. The magnitude, duration and time of appearance of the responses to single stimuli vary within wide limits, and the effect appears and disappears abruptly without any transitional stages. These features of the effect would not be predicted by the hypothesis.

Additional experiments were carried out to test the third point, the effect of the sensitizing drugs on the decay of facilitation. If such an effect occurs, it should appear in the period of enhanced responses before the responses to single stimuli occur. Table 1 shows the result obtained when the effect of tyramine on the decay of facilitation was examined. Clearly, no significant change has occurred. The response to the second stimulus of a pair dies away when the interval between the stimuli is about 3 sec., exactly as in the normal animal. Tests with 933 F gave the same result. In this respect the effective drugs behave in the same limited way as calcium and potassium (Ross & Pantin, 1940), which enhance the response without affecting the rate of decay of facilitation.

Table 1

Interval between stimuli sec.	Height of response to four stimuli (*Calliactis*) mm. on smoked record	
	In natural sea water	After 90–110 min. in tyramine ($1:5\times10^{3}$)
0·5	19·0	35·0
1·0	12·5	23·0
1·5	5·0	10·5
2·0	2·4	5·5
2·4	0·6	1·2
2·6	0·2	0·5
2·8	Trace	0·2
3·0	0	Trace
3·2	0	0

It is clear that the effects on *Calliactis* of drugs like tyramine only partly correspond with the effects expected from truly sensitizing drugs on the facilitator hypothesis. We are left, therefore, with a rather complicated picture. The actinian neuromuscular system shows certain affinities with the sympathetic system of the vertebrates in the class of substances which affect the transmission of excitation to the muscles. But the similarity ends there. The mode of action of the substances on anemones shows that the transmitting system in these animals is very different from the direct chemical system in the vertebrates. These differences are only partly explained in terms of the facilitator hypothesis, which explained satisfactorily the special character of the response to stimulation and the action of ions on *Calliactis*. It must be left to future investigations to explain the anomalous features of the drug effects and provide a clearer picture than we have at present about the mechanism of facilitation.

SUMMARY

1. The action of a number of drugs which affect the neuromuscular systems of vertebrates has been examined on the sea anemone, *Calliactis parasitica*. In contrast to their action in vertebrates, no drugs directly cause contraction in the muscles of the anemone.

2. Some drugs of the group which act at cholinergic junctions in the vertebrates, including acetylcholine itself, were ineffective on *Calliactis*. Atropine and nicotine, of the same group, had depressant effects.

3. Some drugs of the group which act at adrenergic junctions in vertebrates, including adrenaline itself, were ineffective on *Calliactis*. On the other hand, tyramine, tryptamine and 933 F enhanced and sensitized the response to nervous excitation. Thus responses to single stimuli occurred, whereas normally responses only follow the second and

subsequent stimuli. Cocaine enhanced and ergotoxine depressed the responses of *Calliactis* in a manner analogous to their effects in vertebrates.

4. No significant effects were observed with other substances, including strychnine, veratrine, histamine, betaine and tetramethylammonium hydroxide.

5. The limitation of the drug effects to enhancement and sensitization supports the view that neuromuscular transmission in *Calliactis* cannot be a simple chemical process as in the vertebrates. In general the results support the hypothesis that there is a separate process of sensitization which must precede the excitation of the muscle in these animals. On the other hand, the drugs which have sensitizing effects on *Calliactis* do not possess all the properties that would be expected of a natural 'sensitizer' or 'facilitator' carrying out this process of sensitization at the nerve ending.

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FACILITATION IN SEA ANEMONES

II. TESTS ON EXTRACTS

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(*Received* 23 *May* 1945)

(With Four Text-figures)

INTRODUCTION

In previous papers (Ross & Pantin, 1940; Ross, 1945), the view was advanced that a chemical substance, the 'facilitator', is released at the nerve endings in certain muscles in the sea anemone, *Calliactis parasitica*, and carries out the process of neuromuscular facilitation described by Pantin (1935*a*, *b*, *c*, *d*). The object of the experiments described in this paper was to find out if a substance exists in these animals possessing the properties of the hypothetical 'facilitator'.

The experiments are of two types: (1) attempts to detect a substance with facilitating properties in the fluid bathing stimulated anemones; (2) attempts to detect a substance with facilitating properties in extracts of anemones.

TESTS ON SEA WATER BATHING STIMULATED ANEMONES

A number of experiments of the first type were carried out on *Calliactis parasitica* and *Metridium senile*. The procedure was to maintain a number of anemones in a state of continual activity over a period and test the effect of the sea water in which the active animals were bathed on a fresh anemone.

In a typical experiment six anemones were placed in a jar containing about 400 c.c. of sea water and made to contract repeatedly by mechanical stimulation for 12 hr. A seventh anemone was placed in the jar at the same time and stimulated electrically about once every half hour and its response recorded on a smoked drum. No change was observed in the response of this test animal; its size and form remained perfectly normal and there was no tendency to respond to single stimuli. The same result was obtained when the sea water was tested on another animal in another jar not containing the stimulated animals.

All experiments of this type, involving tests on the sea water in which a number of anemones had been stimulated for varying periods, gave completely negative results. It can be concluded, therefore, that if a 'facilitator' does exist, it is not liberated into the surrounding sea water under the conditions of these experiments in sufficient quantities to affect another animal.

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TESTS ON EXTRACTS OF SEA ANEMONES

In the preparation of tissue extracts for pharmacological tests of this kind a number of factors need to be considered. Most substances with neuromuscular effects, e.g. acetylcholine and adrenaline, are extremely unstable and the risk of destruction during the extraction is very great. Therefore, drastic methods must be avoided and temperatures kept as low as possible. It is also necessary for the extract to be as natural as possible and free from foreign substances which adversely affect the animal on which the effect of the extract is to be observed.

After a number of trials ethyl alcohol was found to be the most satisfactory agent for preparing anemone extracts. Attempts to use the trichloracetic and phosphotungstic acid methods were not successful as such extracts had strong depressant effects on the test animals. With alcohol the precipitation of the protein is practically complete and the alcohol can be removed afterwards at low temperatures by distillation *in vacuo*. Moreover, tests showed that small quantities of alcohol (up to 2%) in the sea water have no effect on the anemones, so that any traces that remain will not affect the result. An account of the method follows.

Ten large sea anemones (*Calliactis parasitica* or *Metridium senile*) were ground up in a mincing machine and extracted immediately in 300–400 c.c. cold ethyl alcohol (95%). After standing for 24 hr. or more in the cold, the solid matter, consisting of the insoluble parts of the tissues and the precipitated protein, was removed by filtering. The alcoholic solution was then distilled *in vacuo* until the alcohol had been removed. This caused certain constituents of the extract such as lipoids to settle out and these were removed by centrifuging or filtering. The fluid that is left is then a water extract, usually about 50 c.c. in volume, containing only those substances that are soluble both in water and alcohol.

Many extracts of *Calliactis* and *Metridium* were prepared by the alcohol method. The action of these extracts was tested on both these animals at dilutions varying from 1 : 10 to 1 : 500 parts of extract in sea water. As in the experiments with drugs, the responses of the test animals were recorded at the beginning of each experiment. Then a quantity of the extract was run into the sea water from a pipette and mixed well. After that the response to stimulation and the behaviour of the animal were observed closely. The methods for stimulating the test animal and recording the responses were the same as those used in earlier work on this subject. Stimuli were provided by condenser discharges delivered through Ag/AgCl electrodes and the contractions of the sphincter of *Calliactis* or the longitudinal mesenteric muscles of *Metridium* were recorded on a smoked drum.

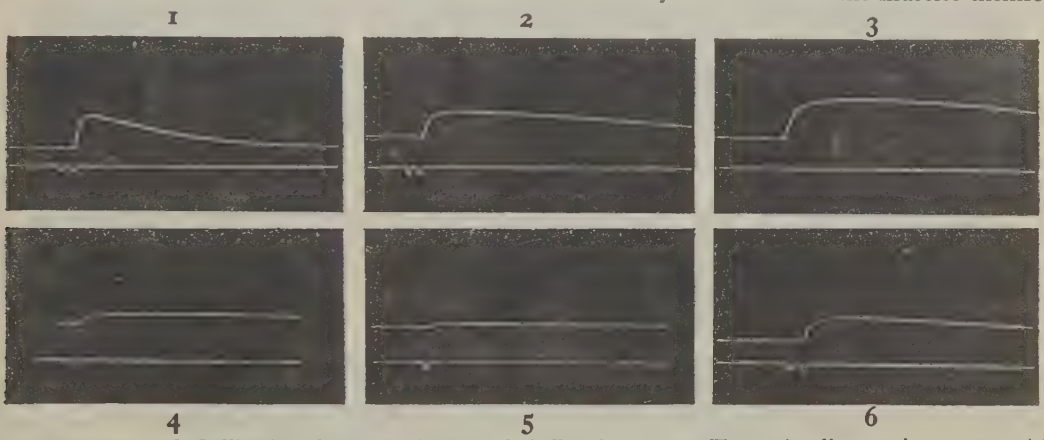

Fig. 1. Responses of *Calliactis* sphincter. Action of *Calliactis* extract. Two stimuli at 1 in 0·5 sec. (1) in natural sea water, (2) after 4 min. in extract (1 part in 100 parts sea water). One stimulus (3) after 5 min., (4) after 9 min., and (5) after 10 min. in extract; two stimuli (6) at 1 in 0·5 sec. after 17 min. in extract. (Note reappearance of response on second stimulus.)

The most definite and consistent property of the anemone extracts is the ability to cause responses to single stimuli. These appeared in a large number of experiments soon after the extract was introduced. About fifty tests were carried out in all and responses to single stimuli were observed in about twenty-five of these. Figs. 1–3 show some of the contractions obtained from *Calliactis* sphincter and the mesenteric retractor muscles of *Metridium* in these tests. It can be seen that the size of the responses to single stimuli varies considerably. Sometimes they are very small, but at times they equal and even exceed the size of the normal response to two stimuli close together.

There can be no doubt that these are truly responses to single impulses and not additional responses due to 'after-discharge' (Pantin, 1935*c*), i.e. the setting up of more than one impulse by one stimulus. If 'after-discharge' were the cause of the effect, a series of stimuli would be expected to give rise to many additional contractions. However, the rule that each effective stimulus causes a single response continues to operate even when the responses to single stimuli are most in evidence. Furthermore, a close examination of the records shows that the latent period between the application of the stimulus and the beginning of the contraction is the same in these responses as in the normal response to the second stimulus. This would not happen if the animal were responding to an additional impulse arriving some time after the first.

When the extract is first introduced into the sea water both *Calliactis* and *Metridium* usually respond with a quick contraction. This contraction only lasts for a short time, less than 1 min. as a rule, and when relaxation is complete the effect of the extract on the recorded response of the anemone can be observed without further contractions of this kind. It is unlikely that this spontaneous contraction is due to an effect by the extract on the muscles themselves. It begins with a twitching of the tentacles which grows in intensity until closure of the animal takes place. This is the usual form of the response to peripheral stimulation. Moreover, if the extract were acting directly on the muscles so as to cause this contraction, the effect would not be expected to be of such short duration or its recovery so complete.

Responses to single stimuli may occur at any time between 1 and 15 min. after the introduction of the extract. Unlike the single shock responses caused by the effective drugs, this effect is seldom preceded by a period of enhanced responses. But like the drug effects, the responses to single stimuli occur suddenly; there is no transition from small to larger responses as the effect develops. The responses to single stimuli soon disappear. Usually after a few minutes the response occurs only after two stimuli as in the untreated animal. After that the extract exerts a depressant action and the response becomes steadily slower and smaller as it does with certain ions and drugs.

Fig. 1 shows the effect of *Calliactis* extract on the response of *Calliactis* at a concentration of 1 part extract in 100 parts sea water. In this experiment the response was unchanged after 4 min. but after 5, 9 and 10 min. responses to single stimuli were recorded. The effect gradually disappeared, the responses to single stimuli becoming smaller and smaller until they vanished altogether after 17 min. This concentration (1 : 100) of *Calliactis* extract proved most effective in causing the effect. Stronger solutions (1 : 10, 1 : 25, 1 : 50) all caused responses to single stimuli, but these were less than half the size of the response of the untreated animal to two stimuli at 1 sec. intervals. At the highest concentrations the extract had marked depressant effects which almost abolished the response after 1 hr., and to a single stimulus 2 min. after the extract was introduced (Fig. 2 B), but the effect only lasted for 3 min.

Extracts of *Metridium* are equally effective in causing responses to single stimuli. Fig. 3 shows the effects of *Metridium* extracts at concentrations of 1 : 50 and 1 : 250. The stronger dose caused the effect within 2 min., but it only lasted 2 min. before it disappeared. At a dilution of 1 : 250 the responses to single stimuli lasted much longer, and although the normal response to the second stimulus predominated after 20 min., single stimuli caused occasional responses up to 50 min. after the extract was introduced.

Apart from the depressant effects which set in after long exposure, the responses to single stimuli are the only consistent effects of the extracts. In

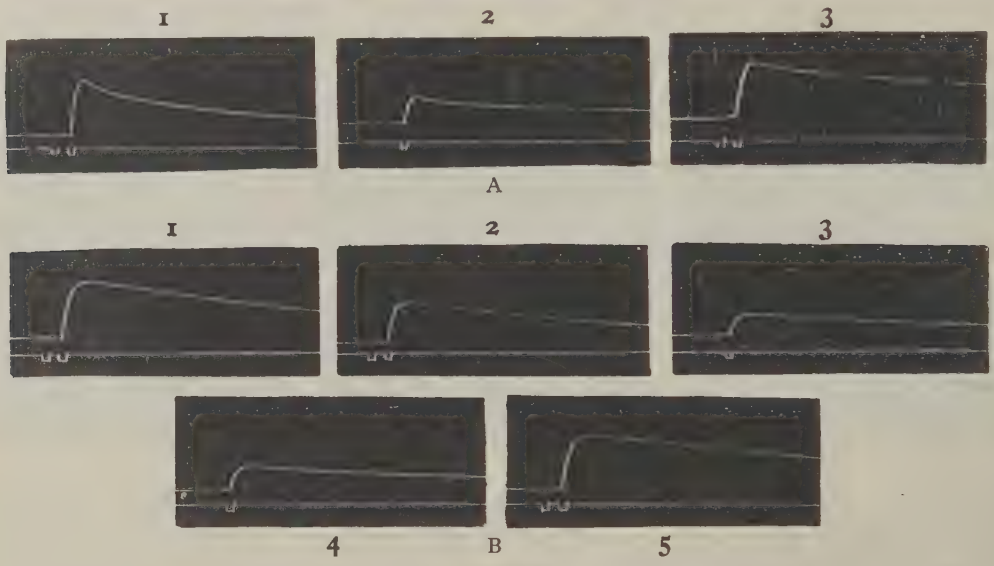

Fig. 2. Responses of mesenteric retractor system of *Metridium*. A. Action of *Calliactis* extract (1 part extract in 25 parts sea water). (1) Two stimuli at 1 in 1 sec. in natural sea water; (2) one stimulus after 1 min. in extract; (3) two stimuli at 1 in 1 sec. after 3 min. in extract. B. Action of *Calliactis* extract (1 part extract in 50 parts sea water). Two stimuli at 1 in 1 sec. (1) in natural sea water, and (2) after 1 min. in extract; one stimulus (3) after 2 min. in extract, (4) after 3 min. in extract; (5) two stimuli at 1 in 1 sec. after 5 min. in extract.

this may account for the poor effects obtained. At dilutions above 1 : 100 the effect becomes more and more difficult to demonstrate and it could not be obtained at dilutions of 1 : 500 and over.

Metridium proved to be a more satisfactory test animal than *Calliactis*. The responses to single stimuli appear more rapidly and as a rule are larger than those obtained from *Calliactis*. Possibly this is because the active substance in the extract can reach the mesenteric muscles of *Metridium* more easily than the sphincter of *Calliactis*. Fig. 2 A shows the effect of a *Calliactis* extract diluted 25 times in sea water on *Metridium*. A response to a single stimulus occurred 1 min. after the extract was introduced, but 2 min. later the animal reverted to normal again. At a dilution of 1 : 50 *Metridium* responded occasional experiments, however, the response was enhanced considerably. This only happened in three out of fifty tests and cannot be regarded as an essential feature of the extract effect. An example of enhancement caused by an extract is shown in Fig. 4. In this case the size of the response was more than doubled by *Calliactis* extract at a dilution of 1 : 20. Unlike the enhancement observed with certain drugs, this effect did not precede but followed the responses to single stimuli, occurring 30 min. after the experiment began. The absence of the marked enhancing action, which was a consistent feature of the effects of those drugs causing responses to single stimuli, suggests that the action of the extract is fundamentally different from the action of the drugs.

The records that have been described show that a substance capable of causing responses to single stimuli exists in sea anemones. It does not follow from these experiments, however, that this substance is the hypothetical 'facilitator', whose existence was postulated in preceding papers. There is no evidence so far which associates the active substance in the extracts with the excitation which should cause the 'facilitator' to be released. All the extracts were prepared from animals which were chopped up into small pieces, a process which must excite what remains of the neuromuscular system intensely. But it is a considerable advance to have demonstrated the existence of a substance with this property in the anemone extracts and opens the way for further investigation.

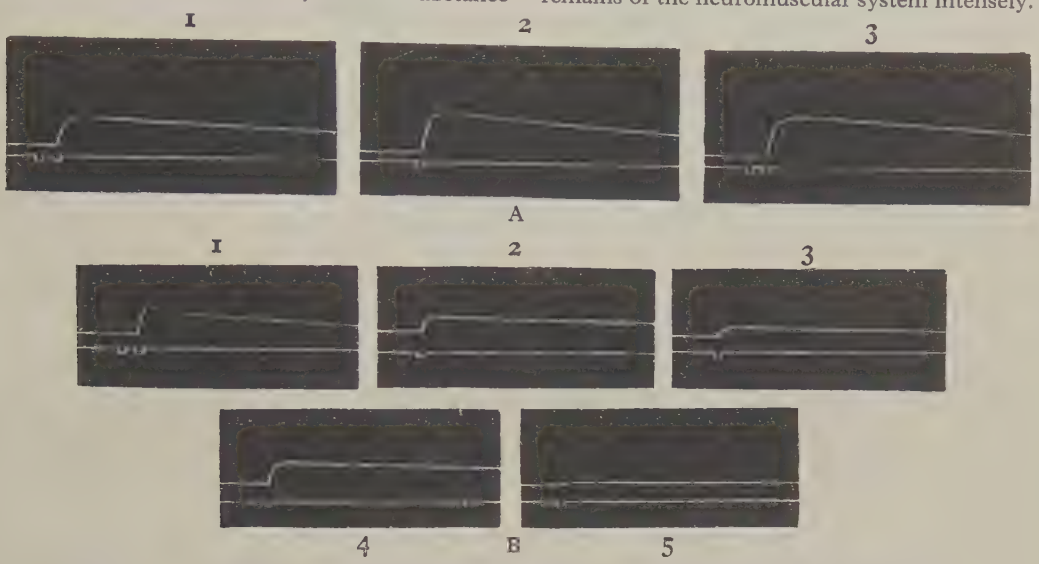

Fig. 3. Responses of mesenteric retractor system of *Metridium*. A. Action of *Metridium* extract (one part in 50 parts sea water). (1) Two stimuli at 1 in 1 sec. in natural sea water; (2) one stimulus after 2 min. in extract; (3) two stimuli after 4 min. in extract. B. Action of *Metridium* extract (1 part in 250 parts sea water). (1) two stimuli at 1 in 1 sec. in natural sea water; one stimulus (2) after 5 min., (3) after 20 min., (4) after 40 min. and (5) after 50 min. in extract.

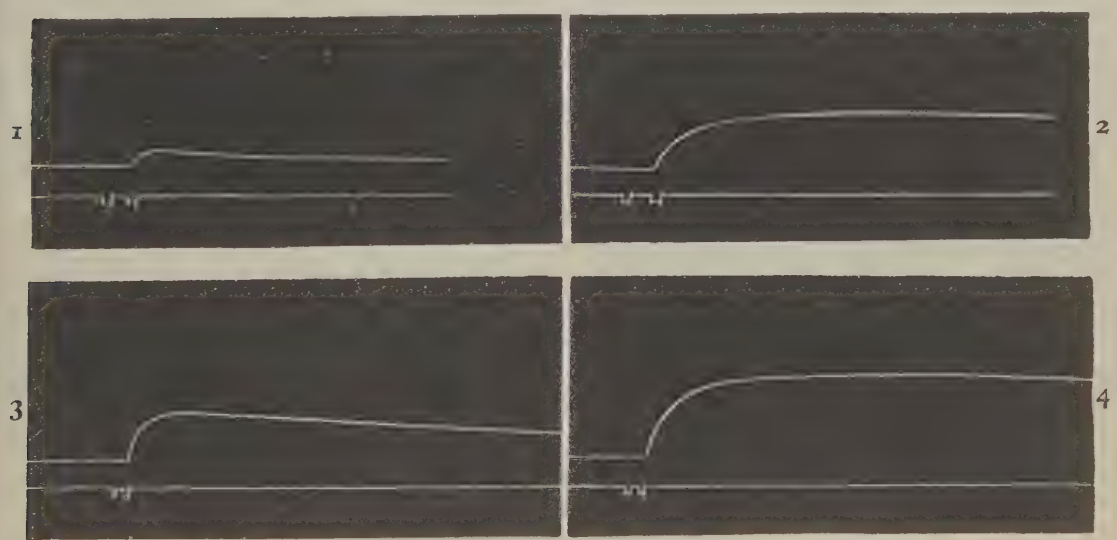

Fig. 4. Responses of *Calliactis* sphincter. Increase in size of response with extract of *Calliactis* (1 part extract in 20 parts sea water). Two stimuli at 1 in 1 sec. (1) in natural sea water, (2) after 34 min. in extract; two stimuli at 1 in 0·5 sec. (3) in natural sea water, (4) after 41 min. in extract.

DISCUSSION

The hypothesis was advanced in previous papers that there are two processes in neuromuscular transmission in sea anemones: (1) a process of sensitization or facilitation, carried out by a 'facilitator' released at the nerve endings; (2) a process of excitation of the muscle which can only occur when facilitation has been set up. Up to a certain point, the action of drugs and ions agreed closely with the predictions of this hypothesis. Under the appropriate conditions they may enhance the size of the responses and sensitize the muscles to respond to single stimuli, whilst they never directly cause contractions of the muscle (Ross & Pantin, 1940; Ross, 1945).

In this paper an attempt is made to determine whether a substance possessing facilitating properties is actually produced in the anemone itself. The experiments show that a substance can be extracted, especially from *Metridium* tissues, which possesses some of the properties of a 'facilitator'. In particular, the presence of extract permits the appearance of a response to a single stimulus and, unlike the corresponding effect with some drugs, it exerts this effect with regularity. It also acts at great dilution, 1 part extract in 250 parts sea water giving a significant response, and acts without much delay, usually after about 5 min., but ranging from 1 to 15 min. in different experiments. These latter features of the extract effect do not correspond with the effects of the drugs, tyramine, tryptamine and 933 F, which caused responses to single stimuli only at relatively high concentrations and after 2–3 hr. It is likely, therefore, that the sensitizing effects of the extract and drugs are due to different substances and they may reflect different modes of action altogether.

The extracts, like the drugs, fail to exhibit all the effects that would be expected of a facilitating substance. The presence of the extract does not delay the decay of facilitation in the expected manner. This point was tested in the same way as it was with tyramine and 933 F. Records were taken of the time required for the facilitating effect of a stimulus to die away in the period before and after responses to single stimuli occurred. In no case did this time exceed the limit of 3 sec. which applies to an anemone in sea water at ordinary temperatures. A parallel failure was found in the action of drugs (Ross, 1945). The extract fails in another respect as well. Unlike the effective drugs, it has no marked effect on the size of the response. Only three out of fifty tests showed any increase in the size of the response and in these cases the increase is not great (Fig. 4), when compared with the enhancement caused by drugs.

We are left, therefore, in the paradoxical situation that both drugs and extract produce some of the effects expected of a facilitating substance, but they neither produce all the expected effects nor do they fail or succeed in the same way. Nevertheless, even though it is not yet possible to account for all the facts, the evidence is clearly against transmission being due to the direct action of some chemical substance on the muscles, and there are sufficient points of agreement to indicate a chemical link in the facilitation process.

SUMMARY

1. Extracts of the sea anemones, *Calliactis parasitica* and *Metridium senile*, have been prepared and their effects on the neuromuscular response of these anemones tested. The presence of extract sensitizes the organism so that a response is given to a single stimulus, whereas normally this occurs only on the second and subsequent stimuli. No other significant effects were observed.

2. The sensitizing effect of the extract differs from the effect of a sensitizing drug like tyramine; it appears more quickly, more regularly, and it is rarely accompanied by an increase in the size of the muscular contraction.

3. The fact that a substance with sensitizing properties has been detected in anemones supports the view that a 'sensitizer' or 'facilitator' exists and takes part in neuromuscular transmission in these animals. Nevertheless, the extract, like the sensitizing drugs, lacks some of the properties that would be expected of a true 'sensitizer' or 'facilitator'.

The author wishes to make the following acknowledgements: to Dr C. F. A. Pantin, F.R.S., under whose direction the work was carried out; to Prof. J. Gray, F.R.S., for use of the facilities at the Zoological Laboratory, Cambridge; to the late Dr Stanley Kemp, F.R.S., for the use of the facilities at the Marine Biological Laboratory, Plymouth; to Dr F. C. MacIntosh, of the National Institute of Medical Research, for advice on methods.

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THE MECHANISM OF LOCOMOTION IN GASTROPOD MOLLUSCS

II. KINETICS

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(*Received* 1 *June* 1945)

(With Thirteen Text-figures)

As is the case in all terrestrial animals, the progression of a snail or other gastropod depends on the exercise by the animal of an adequate force directed posteriorly against the ground; in an animal moving horizontally and at constant speed, this force overcomes the frictional resistance encountered by the parts which are gliding forwards. The movements of the pedal surface of some gastropods have been discussed in a previous paper (Lissmann, 1945). The experiments described below were designed to correlate these movements with the forces set up during locomotion. The salient point which appears to be established is that during progression the foot exhibits (i) areas of forward motion, coinciding with phases of longitudinal muscular contraction, followed by extension; (ii) areas at rest, which remain essentially elongated. However, the state of the muscles is no indication of the tensions they may set up within the body, nor of the propulsive force they may exert; it is by no means obvious how the external forces are produced, or how they lead to progression.

The musculature of the gastropod foot is complex in structure, and there is no general agreement as to how it produces the necessary cycles of longitudinal shortening and elongation on the foot. Whilst there can be little doubt that the process of shortening is effected by the contraction of longitudinal muscle fibres, the subsequent process of elongation is more controversial. According to Jordan (1901), this process is effected by the elastic properties of walls of cavities containing fluid, whilst Trappmann (1916) suggested that transverse muscle fibres were involved. Jordan based his view on experiments with *Aplysia*. He found that vesicles containing body fluid under pressure protruded from isolated pieces of the foot when the latter were made to contract through stimulation. As soon as stimulation ceased the vesicles disappeared and the pieces elongated to their normal length. When the vesicles had been punctured, however, these pieces failed to elongate after stimulation had been discontinued, although the muscles became soft and did not resist passive stretch.

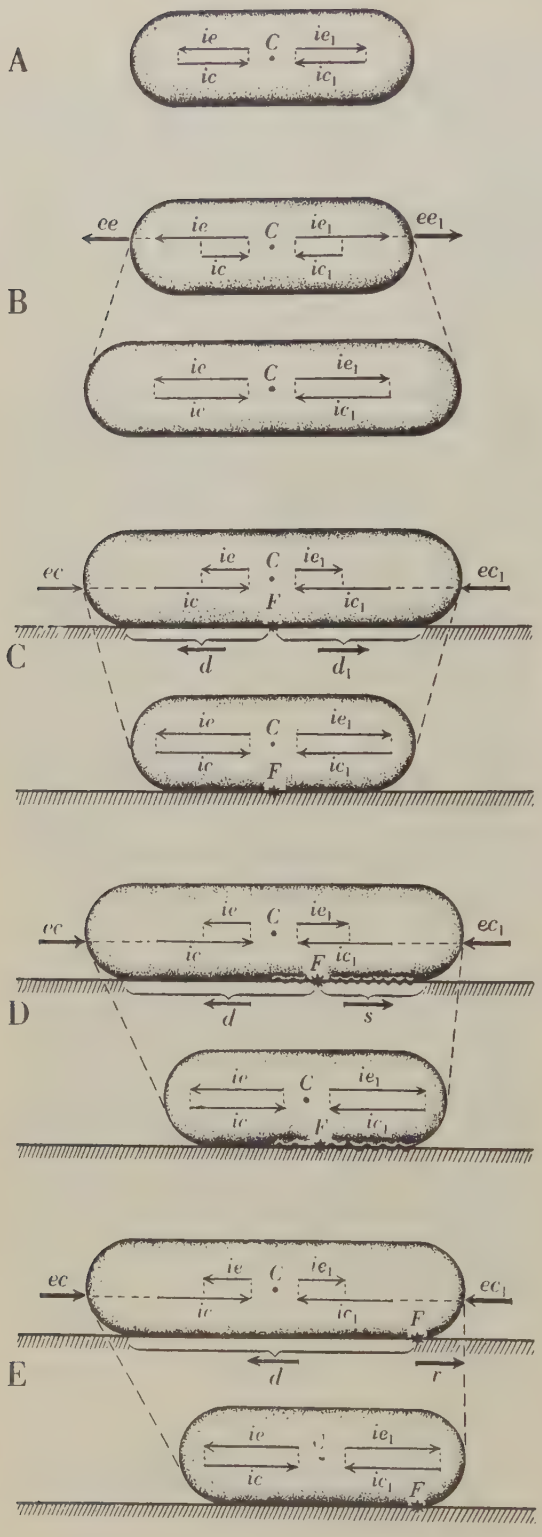
So far as locomotion is concerned *Aplysia* differs markedly from the gastropods discussed here. No attempt has been made in the course of the present investigation to elucidate experimentally the internal mechanism, but some conclusions can be drawn from the observations reported below. With regard to the external mechanics of movement it is immaterial whether antagonism to the longitudinal fibres is effected by hydrostatic pressure, elasticity of connective tissue, or by a set of transverse muscles; the existence of two antagonistic forces—one leading to elongation, the other to longitudinal contraction—is essential, since longitudinal shortening cannot effect a forward movement of the anterior end of the foot, whilst elongation alone cannot produce a forward displacement of the hind end of the foot. Therefore, if the foot as a whole is in steady progression and retains a constant length, both these forces must be operative, and all endeavour to explain locomotion as being due to either one of them alone must fall short of supplying a complete answer.

MECHANICAL PRINCIPLES AND TERMINOLOGY

Before considering the movements of a body which, like that of a snail is without rigid skeletal elements, it is profitable to recall some elementary mechanical principles applicable to such a system. An inflated rubber balloon may conveniently serve as an illustration, especially if Jordan's picture of antagonism between hydrostatic pressure and longitudinal contraction be accepted.

Only forces acting parallel to the long axis of the body will be taken into account, and the following abbreviations be used in the text below:

Internal force of contraction	ic, ic_1
Internal force of extension	ie, ie_1
Internal friction	f
External force of contraction	ec, ec_1
External force of extension	ee, ee_1
Reactions from the ground:	
(1) External sliding friction (drag)	d, d_1
(2) External sliding friction (slip)	s
(3) Static reaction from the ground	r
Point of frictional equilibrium	F



If an inflated balloon be freely suspended, the pressure inside (*ie*, ie_1) and the elastic force of the outer membrane (*ic*, ic_1) are equal and opposite (Fig. 1 A), and as long as both forces increase or decrease in the same sense and proportion no outward movement will occur. However, as soon as only one of these forces is changed a deformation of the body will become apparent. When *ie*, ie_1 is increased, or if *ic*, ic_1 is decreased, elongation will take place; when *ic*, ic_1 is increased, or *ie*, ie_1 decreased, the body will shorten longitudinally. It is assumed that there is a sliding readjustment of the two antagonistic forces, and whenever a change of one of them causes movement, the other automatically undergoes a corresponding change, e.g. if the internal pressure of the balloon be increased, the resultant expansion thereby increases the tension of the outer membrane until a new state of equilibrium is reached (Fig. 1 B). Thus an external force of contraction (*ec*, ec_1) can be expected to operate as long as the resultant vectors converge within the body ($ic > ie$); an external force of extension must exist whenever the vectors point outwards ($ie > ic$). In a complex system like the body of a snail these changes must be significant enough to overcome any internal friction (*f*) which might impede the spreading of the effective force throughout the system. The external forces are therefore

Longitudinal contraction	$ec = ic - (ie + f)$
extension	$ee = ie - (ic + f)$

When either of these two forces operates while the body rests horizontally on solid substratum, the situation is changed owing to the interactions between the body's surface and the ground. These interactions can be expressed as forces acting tangentially against the ground and the surface of the body, i.e. as friction (*d*) which opposes the external force. If the body rests uniformly on the ground and undergoes a uniform contraction, both ends will tend to approach the centre of gravity (Fig. 1 C), and the farther away from the centre of gravity a point is situated, the greater will be its excursion,

Fig. 1. Model illustrating the relationship between the internal forces, the external forces, and the reactions from the ground. A. An inflated balloon; the internal forces of extension (*ie*, ie_1) and of contraction (*ic*, ic_1) are balanced. B. Increase of *ie*, ie_1 causes the appearance of an external force of extension (*ee*, ee_1), and a movement will result until a new state of equilibrium is reached between the internal forces. C. Through increase of *ic*, ic_1, while the model rests on the ground, an external force of contraction (*ec*, ec_1) emerges, causing displacement of the surfaces towards *F* (point of frictional equilibrium). The external forces act against symmetrical external friction (*d*, d_1). D. As in C; through asymmetrical external friction (rough ground under one half), *F* is shifted towards the area of higher friction, which causes a differential excursion of the two ends (drag = *d*; slip = *s*). E. Absolute fixation at one end (*F*) produces a static reaction from the ground (*r*) under *F*, and a displacement of the rest of the body towards *F*.

and the greater its velocity. Only the area vertically beneath the centre of gravity will remain fixed; this point may be termed the point of frictional equilibrium (F).

If during the contraction phase the degree of shortening is uniform, the force exerted by any contracting unit varies more or less inversely with its distance from the point of frictional equilibrium, for the force exerted by the section nearest to F has not only to overcome the friction accompanying its own deformation, but must also exert a tractive force on the more distal parts.

The body will behave similarly when exposed to a force of extension (*ee*), but this time both ends will tend to move away from F, as in Fig. 1 B.

The following general relations between the external forces (*ec*, *ee*) and the external friction (d) can be expected: (i) no displacement will occur when $ec < d$, or $ee < d$, because the external force is insufficient to overcome the initial static friction; (ii) acceleration will result when $ec > d$, or $ee > d$. The component vectors of each force, of *ec* and ec_1, and of *ee* and ee_1, must under all circumstances be equal and opposite, as must be d and d_1 (external friction), and in the case discussed above, the nature of the surfaces and the equal distribution of weight make for a symmetrical appearance of friction and displacement.

If the body undergoes the same deformation as before, while the substrate under one half is smooth and under the other rough, or if the body is fitted with devices which increase the friction under one half, then the point of frictional equilibrium will no longer coincide with the position vertically beneath the centre of gravity, but will be shifted towards the area of higher friction (Fig. 1 D). As before, F will remain stationary and the degree of displacement of any point on the body will vary directly with its distance from F. Since one of the two ends is now farther away from F than the other, it will execute a larger movement. The friction accompanying the larger movement may be termed 'drag', while the smaller excursion at the opposite end is known as 'slip' (d and s in Fig. 1 D).

In the extreme case when friction becomes infinitely great at one end of the body, whereby this remains fixed to the ground, the whole body will tend to approach this area of fixation when a longitudinal contraction takes place (Fig. 1 E), and will tend to move away from it in case of elongation. In both cases the maximum distance of displacement will be twice that of the original movement when the point of frictional equilibrium was situated midway between the two extremities, because F is now situated twice as far away. In other words, one of the two component vectors of *ee*, ee_1 or *ec*, ec_1 has become completely neutralized by a static reaction from the ground (r), and the displacement has become unidirectional.

So far tensions and thrusts have been conceived as developed between two surfaces exerting sliding friction, or between one such area and a fixed point. Additional forces can also be set up between two fixed points; such instances will be considered later (p. 42).

In gastropods the external forces, which are the actual propulsive agents, are derived from an interplay of internal forces generated through co-ordinated muscular activity. At any one moment the magnitude and direction of the external forces must be essentially considered as a balance between the horizontal longitudinal components of the internal forces of extension and of contraction, reduced by a certain amount of internal friction.

Fixation to the ground is assumed to be effected by adhesion of longitudinally relaxed parts of the sole by means of mucus (Parker, 1911). Unlike the instances discussed above, or unlike the leech, the whole area of fixation is not static; each point within that area remains stationary relative to the ground for a brief period, but the central point of the area of fixation shifts continuously and with the same velocity as that of the preceding (anterior) and the following (posterior) longitudinal contraction.

Detachment and reduction of friction may be aided—as Rotarides (1927) suggests—by expulsion of mucus from the numerous glands usually distributed on the sole. This may be caused directly through the muscular contraction. The mucus may therefore play a double role; as adhesive and as lubricant. Frequently a simultaneous contraction of longitudinal and dorso-ventral fibres is taken for granted, because the regions of longitudinal contraction are lifted off the ground. There is no need for this assumption on mechanical grounds; any local increase in tension through muscular contraction will produce the same effect, while a reduction in tension will produce a bulge, just as a bulge will appear on the weakest part of a tyre under high pressure.

It is tempting to attribute the internal pressure to the longitudinal contractions. However, while these contractions undoubtedly have an effect, it must be remembered that the internal pressure of the system cannot be greater than the weakest point of the balancing tension of the enclosing walls. Whether the walls of the system are exclusively represented by the 'vesicles' described by Jordan, or whether there is a general pressure or pressure gradient within the body spaces remains doubtful. This point is of importance in considering the internal friction.

RELATIONSHIP OF INTERNAL AND EXTERNAL FORCES AS DEDUCED FROM *POMATIAS ELEGANS*

The foregoing considerations can now be exemplified by a concrete case. From a mechanical point of view

the conditions in *Pomatias elegans* appear reasonably clear. Although this species presents problems of its own and does not lend itself readily to experimental enquiry, it is more suitable than the snail for illustrating the general distribution of forces, and may thus serve to elucidate the somewhat complex phenomena discussed in the subsequent analysis of *Helix*. As has been suggested previously the physiology of locomotion of the two genera reveals some fundamental similarities despite a strikingly different appearance.

When an animal like *Pomatias* is at rest it can be assumed that the muscular tone is balanced, and whatever the magnitude of the internal forces (*ic*, *ie*) may be, they must be either equal and opposite

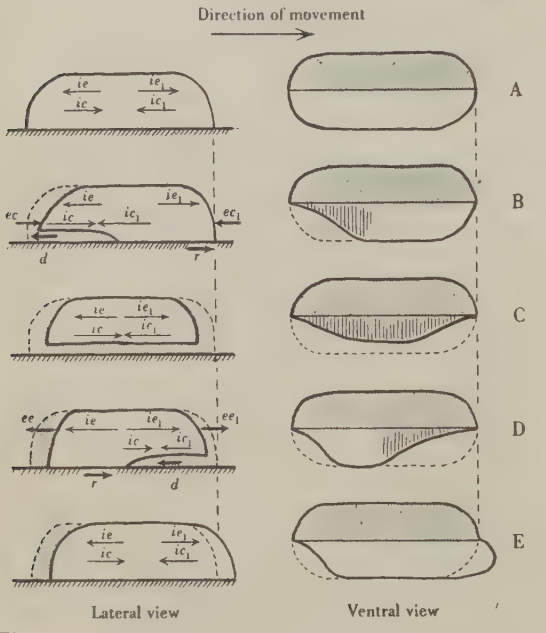


Fig. 2. Diagrammatic representation of the forces leading to locomotion in *Pomatias elegans*; *ie*, ie_1 internal pressure; *ic*, ic_1 longitudinal contraction; *ec*, ec_1 and *ee*, ee_1 external forces of contraction and extension; *d* = dynamic friction; *r* = static reaction from the ground.

(Fig. 2 A), or their difference must be smaller than the external friction between the animal and the substrate:

$ie = ic$, or

$ie - ic = ee < d$ (long. thrust) } stress on substrate
$ic - ie = ec < d$ (long. tension) } without displacement.

When a wave of contraction starts at the posterior end of the foot, this region of contraction is lifted from the ground and, owing to the shortening of the muscles, it is drawn forward towards the anterior regions which, being still relaxed, adhere firmly to the ground. The tension set up between the adherent and non-adherent regions is undoubtedly due to a contraction of longitudinal muscles. The external posteriorly directed force (ec_1, Fig. 2 B) generated by these muscles is resisted through interposition of a zone of relaxed tissue, by an equal and opposite reaction from the ground (*r*). The anteriorly directed component of the contraction (*ec*), on the other hand, accounts for (i) a forward acceleration of mass (quantitatively negligible); (ii) any frictional resistance that the forward gliding posterior end may have to overcome, including any adhesive forces (*d* in Fig. 2 B). It can also be assumed that this contraction exerts a pressure on the body fluid and contributes to driving it into the other stationary half of the foot.

As the contraction passes forward the area of the anteriorly situated adhering surface is reduced (partly by detachment and partly by reduction of its transverse expansion); eventually the area of the adherent region is insufficient to resist the force of longitudinal muscular contraction. At this stage a backward slip of the anterior end occurs. When the phase of contraction occupies one half of the whole foot the complete half foot is, as a rule, lifted off the ground in a fully contracted state (Fig. 2 C). Simroth (1882) suggested that after the half foot had been lifted from the ground it was advanced relative to the head of the animal by the contraction of caudal muscles. This would however involve a reaction against the ground by the other adherent half foot or by the proboscis, since these now carry the whole load. Simroth's assumption, however, does not seem legitimate in view of the fact that the raised half of the foot is merely brought alongside the relaxed half, and is in no way protracted.

There is no evidence to suggest that in *Pomatias* there may exist a direct antagonism between longitudinal and transverse fibres, because the longitudinal contraction occurs simultaneously with the transverse contraction. This, quite to the contrary, probably means that whilst a simultaneous contraction of longitudinal and transverse fibres takes place in one half foot, a corresponding relaxation of both sets of muscles occurs in the other half. The significance of this suggestion should not be exaggerated, since it must be remembered that in *Haliotis* the conditions appear to be reversed. It may be that in *Pomatias* these synchronous contractions involve a movement of the common centre of gravity towards the middle line of the stationary half foot; this would increase the stability of the animal, just as a bipedal vertebrate tends to shift the centre of gravity towards the supporting limb during slow ambulation.

When one half foot of *Pomatias* is lifted, relaxation begins posteriorly, and the posterior edge of the foot is lowered. As soon as this process has brought a sufficient area of surface into contact with the ground, adhesion to the ground is firmly established (Fig. 2 D). Internal pressure, or other force of elongation (*ie*, ie_1) now increases while the force of longitudinal contraction (*ic*, ic_1) decreases; in this

way the foot regains its original length. The position of the foot relative to the ground is however changed; the posteriorly directed component of the external forces (*ee* in Fig. 2 D) is resisted by the adhesion to the ground of the posterior edge (*r*), whereas the posterior surface of application, and the contraction must be considered as a necessary preparatory act.

The difference between the initial short step and a full step during normal ambulation is diagrammatically shown in Fig. 3. In the latter case the

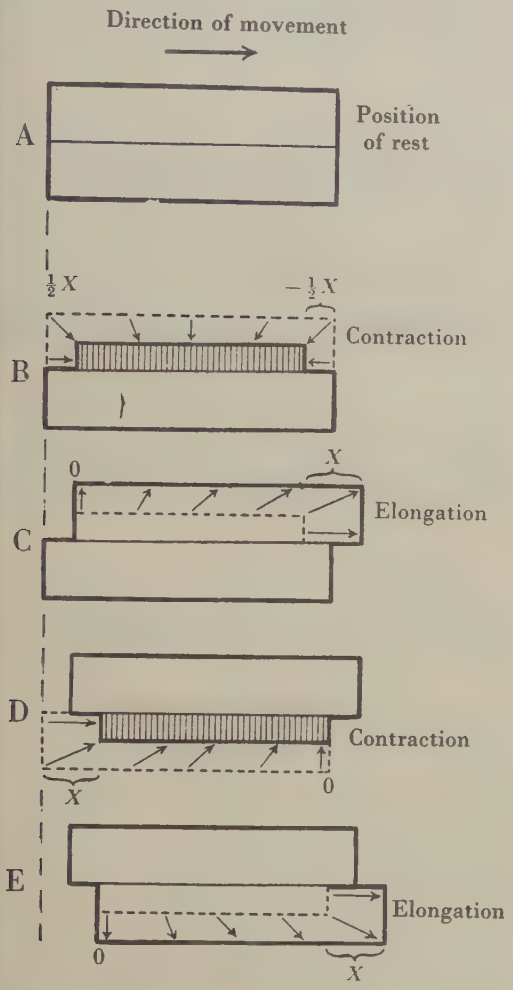


Fig. 3. Diagram showing the difference between the initial short step (A–C), and a step in normal ambulation (C–E) in *Pomatias elegans*. X=length of one step.

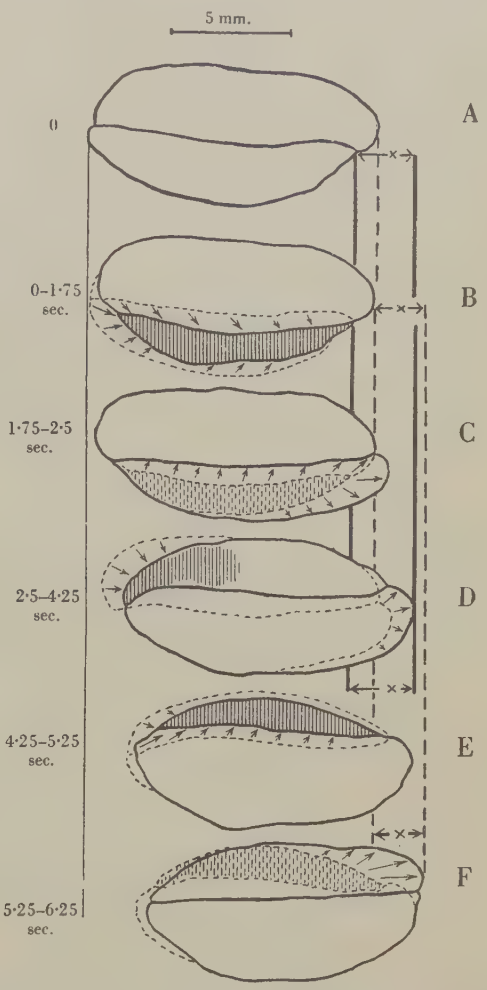


Fig. 4. Ambulation of *Pomatias elegans* (ventral aspect) as recorded by cinematography. The interrupted outline shows the immediately preceding position of the sole. The arrows indicate the direction of movement of the surface; any friction operates in the opposite direction. In D the final phase of elongation is shown anteriorly on the left half foot, and the beginning of the contraction phase on the posterior end of the right half, indicating that the waves of activity spread over the sole in a postero-anterior direction. X=length of one step.

partially contracted anterior region, being out of contact with the ground, is pressed forward, overcoming (by the component ee_1) any frictional resistance (*d*) it may encounter with the substratum.

It will be noted that during this initial short step the mass of the half foot as a whole is not moved forward by energy derived from longitudinal muscular contraction. During this phase the posterior end moves forward, whilst the anterior end moves backwards, i.e. the foot merely shortens. The short step forward is executed exclusively through the force of extension acting against a contraction of longitudinal fibres moves the whole mass of the foot from a posterior position to the level of the stationary half foot, while the force of extension drives it an equal distance forward, as is shown in Fig. 4. Obviously so far as locomotion of this type is concerned, the end from which the

contraction spreads over the foot is of little consequence provided the stationary half foot is firmly fixed to the ground. Forward motion, however, can only be achieved when relaxation begins at the hind end.

The spread of the phase of contraction from the posterior end is probably more advantageous since it tends to relieve at an earlier moment any stress caused by the asymmetrical posture, and permits a larger anterior area of the foot to remain fixed to the ground while the posterior end is brought forward. It seems likely that the asymmetrical position of the two half feet involves stresses between them because the movement of the active half foot affects, to a small extent, the stationary half foot; e.g. the contraction of the active half foot protracts somewhat the posterior edge of the relaxed half foot (Fig. 4 B), while relaxation of an active half foot tends to move forward the anterior margin of the other half foot (Fig. 4 D). It also seems likely that the inactive half foot assists the active half foot in some phases of contraction and extension by enlarging the anterior (or posterior) area of fixation.

ANALYSIS OF FORCES IN *HELIX*

The sole of the snail (*Helix pomatia*) in locomotion has an average surface area of 15·75 cm.² and measures 7·5 cm. in length.* It is divided transversely by eight to ten distinct phases of muscular activity, each of which is separated from the succeeding one by a stationary elongated region. Thus each zone of fixation is bordered anteriorly by an area undergoing extension, while behind it is an area of muscular contraction, and the whole pattern shifts continuously forward. So far as the state of the muscles is concerned any one of the functional units may be considered as comparable to the system described above for *Pomatias*; the simultaneous existence of more than one area of fixation however makes it illegitimate to consider the tensions and thrusts as being solely developed between an area of fixation and an area exerting sliding friction. In the snail, additional forces may be developed between adjacent fixed points. Displacement of a localized area between two fixed points can be effected in a number of ways by different combinations of forces (*ec* and *ee*); these possibilities are illustrated in Fig. 5. But as in all types of animal locomotion where at any one time some part of the body remains fixed to the ground, both the force of extension and the force of longitudinal contraction in the snail must necessarily contribute in equal measure to the distance of forward displacement of either the whole animal or some of its parts. The magnitude of the two forces, however, need not be the same, and will probably depend largely on the position of the point of frictional equilibrium, and the centre of gravity.

* Corresponding values for *H. aspersa*: 4·1 cm.² and 5 cm. length.

If these assumptions and the picture given above for *Pomatias* are correct, and are applicable to the snail, it should be possible to demonstrate experimentally four and possibly five forces: (1) an external force leading to elongation; (2) an external force leading to longitudinal contraction; (3) sliding friction (drag), acting in a posterior direction and developed by regions of the foot which are in forward

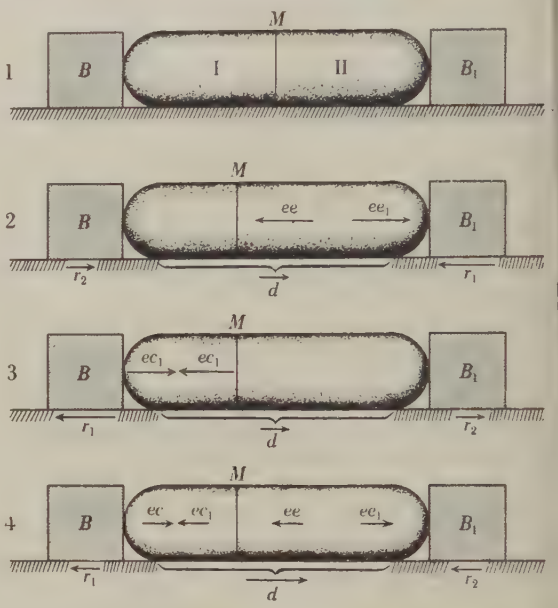


Fig. 5. Model illustrating three different modes of distribution of forces during an active forward displacement of a localized area, situated between an anterior and a posterior area of fixation (B, B_1). (1) A rubber balloon consisting of two chambers (I, II) separated by a median wall (M) is attached to two blocks B, B_1 which rest firmly on the ground; no longitudinal forces are exerted. (2) If chamber II is inflated, it will elongate, thereby compressing chamber I, and developing a longitudinal thrust between B and B_1. M will be displaced towards B. (3) If chamber I is deflated it will contract, thereby extending chamber II, and setting up a tension between B and B_1. M will be displaced towards B. (4) If chamber I is deflated to the same degree and simultaneously as chamber II is inflated, a similar displacement will take place, and both areas of fixation will produce static reactions from the ground acting in the same direction; these two forces balance the friction resulting from the displacement.

motion; it is independent of the nature of the propulsive force; (4) a static reaction from the ground, acting in an anterior direction under the areas of fixation; (5) possibly static tensions and thrusts developed between successive areas of fixation.

External forces. An indication of the nature and magnitude of the forces existing at any one region of the pedal surface of a snail can be obtained by

means of an apparatus previously described (Gray & Lissmann, 1938) which consists essentially of a fixed platform separated by a narrow gap from a movable bridge, the latter being mounted on knife-

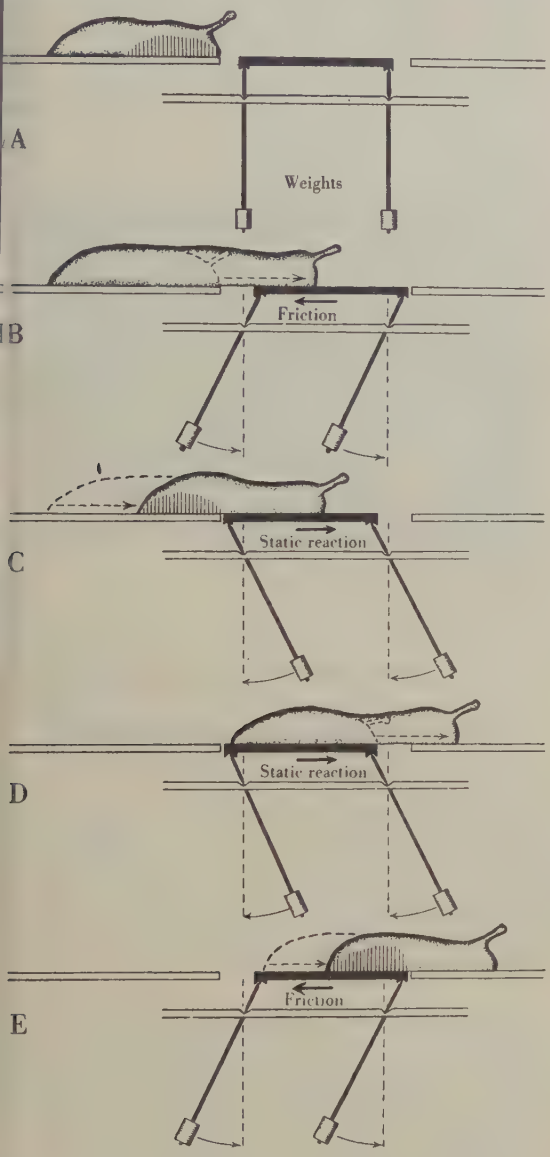


Fig. 6. Diagram illustrating the recording mechanism in some characteristic stages. The displacing force which is applied to the movable bridge reaches a position of equilibrium when the applied force is equal and opposite to the restoring couple of the weights which tend to return the bridge to the position shown in A.

edges in such a way as to record either tensions or thrusts acting between the platform and the bridge while the snail traverses the gap. It is obvious that only a force of extension can lead to a widening of the gap, and only a force of longitudinal contraction can narrow the gap. This is illustrated diagrammatically for four characteristic stages of progression in Fig. 6, each of which corresponds to the changes indicated in Fig. 2 from position A to B, and from position D to E. Interpreted in terms of the foregoing analysis of movement of *Pomatias elegans* this means that when the snail moves from the fixed platform on to the bridge: (i) the gap widens (Fig. 6 B); this can only be due to a force of extension acting against a posterior surface of application; the force recorded represents sliding friction between the part of the animal which is thrust forward and in contact with the bridge; (ii) the gap narrows (Fig. 6 C); this is an indication of a force which leads to longitudinal shortening. The force thus recorded signifies that anteriorly the ground has to resist a backward displacement (static reaction) when the animal has established an anterior fixed point against which posteriorly situated parts are protracted. When the animal moves from the bridge on to the fixed platform: (iii) a static reaction from the ground under a posteriorly established point d'appui is recorded (Fig. 6 C); (iv) Fig. 6 E shows the recording of friction exerted by the posterior part of the body sliding forward.

These four stages show the maxima of tensions and thrusts. As the waves of muscular activity pass over the gap, the magnitude of the forces acting across the gap decreases, corresponding to the decrease of external resistance.

Additional forces developed as static reactions between successive fixed points in *Helix* can be revealed by the same method.

Typical illustrations of records made by the passage of snails (*Helix pomatia* and *H. aspersa*) are shown in Fig. 7. It will be noticed that the passage of the anterior end of the foot on to the bridge is accompanied by a forward thrust from the region of the foot still in contact with the fixed platform—the gap widens. Subsequent to this, however, the tracing rises above the base line and remains so not only while the rest of the animal is moving on to the bridge but also after the whole animal has passed over the gap, thus indicating a tension across the gap—which narrows. The existence of a residual tension after the whole animal is on the bridge is due to the presence of a film of mucus acting across the gap, and introduces a complication in the interpretation of the whole tracing. It is, however, clear that, after the initial thrust, and, as long as the central and posterior regions of the pedal surface pass over the gap, definite tensions are acting between the bridge and the platform. If it be assumed that the tension of the slime band remains constant throughout the whole tracing, this mucus tension must increase any apparent muscular tensions across the gap and must act in opposition to any thrust developed between the bridge and the platform. Therefore, for a true representation of external muscular forces the base line must be

adjusted by an amount equal to the mucus tension recorded after the passage of the animal (Fig. 7). The general conclusion to be drawn from tracings of this type is that the propulsion of the front end of the animal is effected by regions of the foot lying posterior to itself, whereas the hind end is being aided by a pull from regions lying more anteriorly. A continuous but fluctuating thrust acts between the anterior region and the central region, and a continuous tension is developed between the central region and the hind end. The longitudinal muscular contractions (dark waves) which occur throughout the whole length of the foot coincide with these fluctuations. Their effects are usually clearly superimposed on the thrust-tension curve, although they

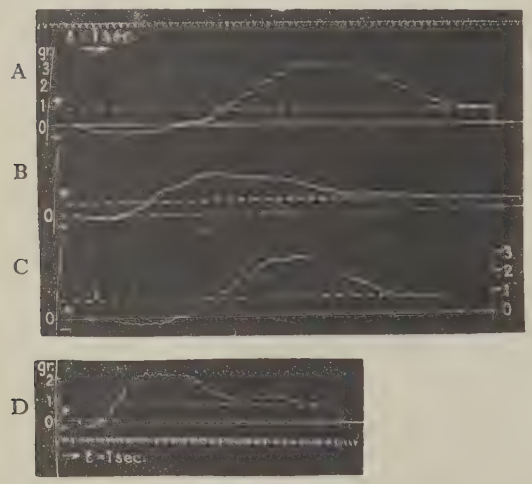


Fig. 7. Tracings showing direction and magnitude of the forces (−forward thrust; +tractive force) recorded by (A–C) *Helix pomatia*, (D) *H. aspersa* moving from a horizontal glass plate on to the recording bridge. Note that after the snail has moved over the gap an appreciable tension persists, which is caused by the mucus band deposited on the track; the interrupted line indicates the magnitude of the mucus tension at the posterior end of the animal. In *H. pomatia* tension and thrust amount to about 2·5 g., in *H. aspersa* about 1 g.

do not affect the direction of the resultant force. Consequently they appear isotonic anteriorly and isometric posteriorly. It follows that those parts of the anterior regions which glide forward are pushed from behind; this thrust not only compresses these parts which shorten isotonically, but also involves a static reaction by the preceding area of fixation (Fig. 5, *2*). Similarly the tensions under the hind end not only overcome the frictional resistance of the forward gliding parts but also exert a tension between two succeding fixed points (Fig. 5, *3*). This conclusion is supported by the observation that the regions of the foot lying posteriorly may be in continuous forward motion, whereas the central and slightly more anterior regions exhibit occasionally a backward slip. The exact proportion of these regions may possibly depend on whether the animal moves vertically or horizontally, and may, during horizontal movement, also depend on the position of the shell, which is not always constant. The relatively complex system thus recorded can best be illustrated by means of a model (Fig. 8). An inflated elastic balloon rests on the ground, adhering firmly by its central region; if then either the hind end (*BM*) contracts, or the front end (*AM*) relaxes, or if both these processes take place simultaneously, the whole surface of the balloon which is in contact with the ground will glide forward with the exception of the central, fixed area. Anteriorly a thrust will be developed, while posteriorly a tension exists between the area of fixation and the forward gliding parts. If

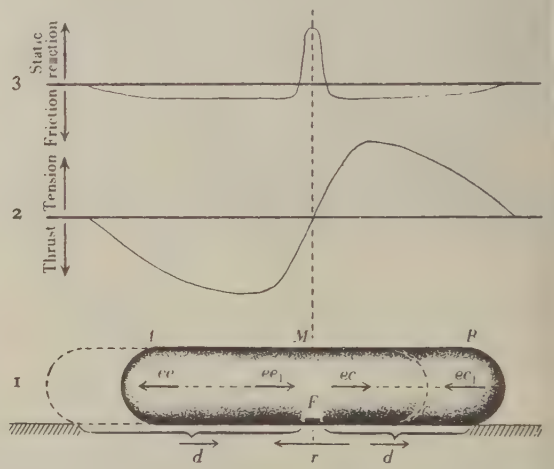


Fig. 8. Model illustrating the mechanical effects of the snail's foot. A rubber balloon rests on solid substrate with its central region firmly attached. If the posterior end (*BM*) contracts, or the anterior end (*AM*) relaxes, a movement will occur leading to the position indicated by the dotted outline. During this process the thrust-tension curve would be essentially similar to that recorded by the snail. *F*=area of fixation.

measured with the apparatus described above a thrust-tension curve can be expected similar to that shown in Fig. 8, *2*; the curve crosses the base line through the area of fixation. The regions behind and in front of the area of fixation exert a sliding friction (*d*) against the ground, while the ground resists a backward displacement with a static reaction (*r*) under the area of fixation itself (Fig. 8, *3*).

Reactions from the ground. The existence of static and dynamic reactions from the ground (*r* and *d*), opposite in direction, can be demonstrated under the sole of a snail in locomotion. As in the model, the former are set up under regions of fixation, the latter under the parts in forward motion.

In both species of snail the areas of fixation on the sole (light transverse bands) are about 5–8 mm. wide. It is therefore possible to demonstrate the static

reaction under these regions by replacing the movable bridge by a glass strip 5–8 mm. wide; maximum backward displacement being obtained—as in the model—at the point where the thrust-tension graph crosses the base line. This was found to be the case for both species (Fig. 9).

When the snail moves over a movable glass strip of appropriate dimensions, mounted between two fixed platforms, and its anterior region has reached the second platform the strip is continuously pushed

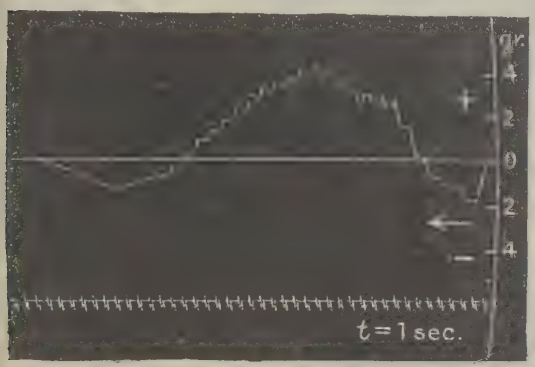


Fig. 9. Typical tracing showing the forces exerted by the foot of *Helix pomatia* on a recording glass strip 7 mm. wide. Note that the maximum force of backward displacement (static reaction +) acts in the central regions of the foot.

a residual tension, owing to the formation of a mucus band connecting the glass strip on either side with a fixed platform, thus producing symmetrical tensions.

An indication of the accuracy of this experiment can be gained by demonstrating the dependence of the magnitude of the static reaction on the degree of external resistance which the animal has to overcome. This external resistance can be increased by a known amount by allowing the animal to move horizontally over the recording glass strip while at the same time it is made to lift a weight over a pulley attached by a string to its shell. Apart from the propulsive forces required to move the animal along, the forces must be increased in order to lift the weight against gravity. This additional force is applied through the areas of fixation and must increase the tangential static reaction from the ground. The greater the load, the greater the reaction (Fig. 10), and the increase of work done under different loads can be calculated from the area of the tracings; it agrees, to a surprising extent, with the theoretical requirements. However, the exact quantitative estimation of the external work which the unloaded animal has to do while creeping horizontally, is considerably complicated by mucus tensions and other factors; it will be referred to in a subsequent publication.

The absence in the central part of these tracings of any sign of the sliding friction (opposite in direction

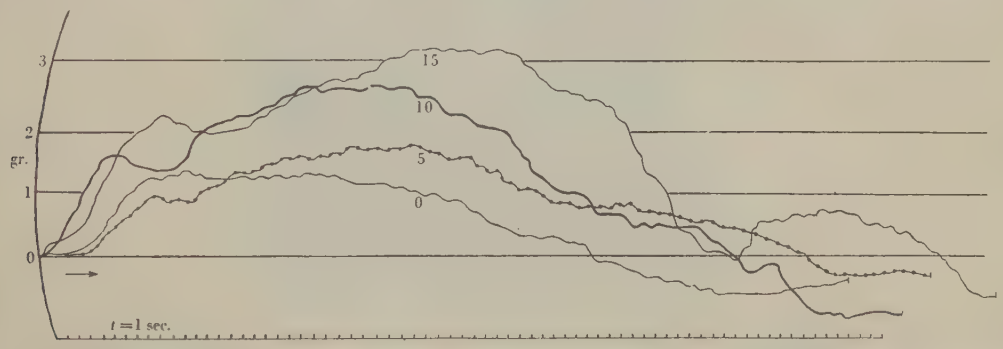
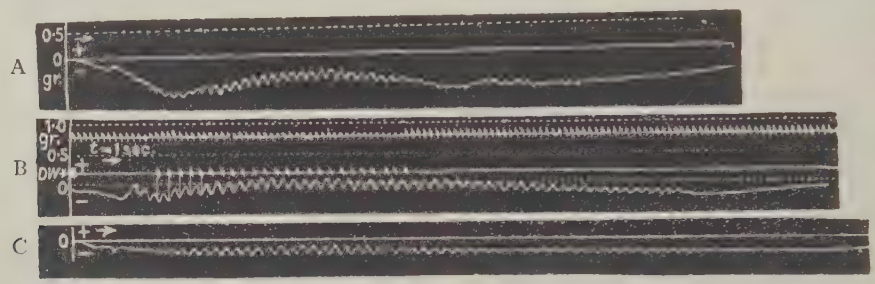


Fig. 10. Tracings showing the force with which a 5 mm. wide recording strip is pushed backwards under the central parts of the sole of *Helix pomatia* and the increase of this 'static reaction from the ground' by letting the animal lift a load while crossing the recording mechanism. The figures 0, 5, 10, 15 indicate the load in grams.

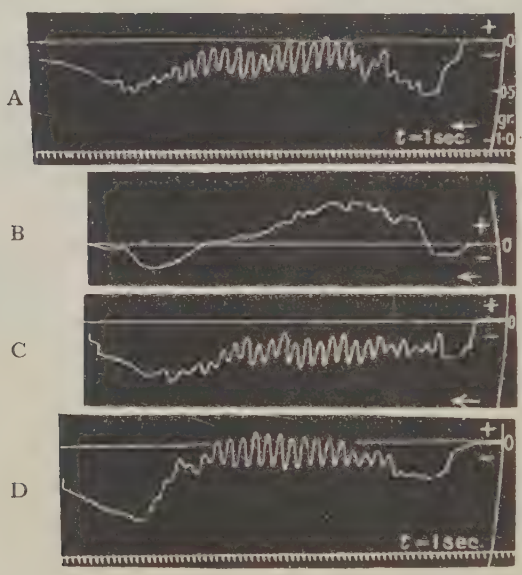
backwards; the force of displacement reaches its maximum when the central region of the foot is on the strip; thereafter the force gradually decreases. During the initial stages, when the snail moves on to the glass strip, the record does not differ markedly from the initial part of the tracing obtained in the bridge experiment, i.e. the strip is pushed forward. Similarly, the strip is pulled in the direction of the animal's motion when its posterior tip moves off the strip, with its anterior and central parts well on the second platform. However, after the animal has crossed the second gap, there is usually no sign of to the static reaction) which can be clearly demonstrated by a similar experiment in the earthworm, may appear puzzling at first sight. But contrary to the conditions in the earthworm the areas fixed to the ground are, in the case of the snail, wider than the forward gliding parts. If the glass strip is 5–8 mm. wide, there will be no stage at which an area of fixation does not either occupy the whole width of the strip or when it does not connect the strip to one of the two fixed platforms. In the former case the strip is displaced backwards, in the latter it remains stationary.

The width of the forward gliding parts is normally 1·5–4 mm. If a movable glass strip of this width is mounted between the two fixed platforms, and its displacements recorded while a snail moves across it, the tracing is of an entirely different character as compared with those obtained with a wider strip: whenever a dark wave at any point along the foot

Bonse (1935). Snails were made to creep over a glass roller of 2 mm. diam. mounted between two glass plates, and the movements of the roller were recorded. The interpretation of these oscillations is, however, based on a number of misconceptions. Neither of the authors recorded the base line, but from the available data it seems legitimate to conclude that they too



I.



II.

Fig. 11. Tracings obtained from two snails (I, II) crossing a narrow strip. As long as the recording strip over which the snails move is about 1·5–4 mm. wide it will be essentially displaced in the direction of the animals motion (→). The displacing force appears greatest during, or just prior to, the passage of the dark waves (regions of forward motion), and is therefore assumed to represent friction. I. Successive records from one specimen of *Helix pomatia* moving slowly across a recording strip (A) 1·9 mm.; (B) 3·2 mm.; (C) 1·9 mm. wide. Note that the whole tracing is beneath the base line in A and C. In B the passage of the dark waves over the recording strip was marked with a tapping key (DW). II. Another animal (moving fast) crossing a recording strip (A) 4 mm.; (B) 7·5 mm.; (C) 4 mm.; (D) 4 mm. wide. Note the appearance of a static reaction (+) in B.

traverses the strip, the latter is dragged in the direction of motion, returning after the passage of the wave more or less to the base line, but more often the whole tracing is beneath the base line (Fig. 11). It appears evident that these displacements are due to sliding friction. Similar experiments, designed to demonstrate the propulsive forces, have been carried out by ten Cate (1922) and repeated by

recorded the sliding friction of the forward gliding parts. Both authors noticed that the recorded deflections do not coincide exactly with the phases of the waves passing over the roller: the explanations offered are, however, in no way convincing. Moreover, the fact that the whole tracing is usually beneath the base line requires an explanation.

While the details of these recorded effects may be

imperfectly understood, the following considerations should be taken into account. The surface of a snail's foot alternately glides over the glass strip and then rests, etc. When a similar experiment is performed by towing an inactive body intermittently over the recording strip between two fixed platforms, the following points emerge: when the body is placed across the strip no horizontal force is recorded. As soon as it is being passively pulled, initially a large 'static friction' is recorded, i.e. a critical value of tension must be reached before the two surfaces (strip and body) begin to glide past each other. When this point has been passed a smaller 'sliding friction' will persist as long as the body is in motion. When the towing is interrupted, a force corresponding more or less to the sliding friction will still be indicated. In the next phase, when the body is pulled again, the 'static friction' will reappear. In this case, too, the whole tracing is beneath the base-line, and the fluctuations merely represent a difference between sliding and static frictions. The larger 'static friction' is recorded just before the body begins to move, not unlike the observations reported by ten Cate and Bonse. While this interpretation may appear plausible in case of passive towing, considerable complications must arise in the case of the snail owing to the visco-elastic properties of the mucus, the vertical displacements of the pedal surface, and the gradual spreading of regions of static and sliding friction on to the recording surface, both of which are preceded and followed by stationary regions exerting forces between them.

In considering the mechanical effects of the snail's foot on the substrate we may conclude from these two types of experiment that the sole is subdivided into narrow transverse regions each exerting friction against the ground, and each bordered in front and behind by wider stationary transverse areas which exert static reactions against the ground; these latter forces, however, vary in magnitude and direction under the various parts of the sole. The difference in width of the two functional regions makes it difficult to record the opposite effects of friction and static reaction in a single experiment. When the recording strip is sufficiently narrow it registers only the sliding friction exerted by the narrow, dark bands. When the width of the strip exceeds a minimum value the friction of the narrow dark bands is not recorded, because the strip is locked to both the fixed platforms by areas of the foot which are in the relaxed state.

By careful adjustment of the width of the recording strip (usually 2·8–5 mm.) it is possible to obtain an intermediate condition under which both sliding friction and static reaction can be recorded. Under such conditions small oscillations appear on the tracing above and below the base line; backward displacement of the strip occurs during the passage of light, relaxed bands. This is particularly obvious when records are made from regions halfway along the foot (Fig. 11, I, B). When the anterior end of the animal passes over the recording strip the records show an alternation between sliding friction and forward thrusts, whilst records from the hind end of the animal indicate sliding friction and static tension.

In all cases the static and dynamic ground reactions as shown in the records must be corrected for the disturbance due to mucus tension acting between the fixed platforms and the recording strip.

DISCUSSION

As the longitudinal contractions on the sole of the snail are the most conspicuous phenomena during locomotion, it is tempting to suggest that these contractions, as they move anteriorly, squeeze the body fluid in that direction, thereby producing a high internal pressure in the anterior region. Such high pressure would tend to elongate the anterior regions. As long as these regions adhere to the ground, the force of extension will merely produce a longitudinal thrust and a corresponding static reaction from the ground. However, when the longitudinal contractions pass forward and thus involve detachment of localized regions in the anterior half, the latter elongate and are displaced forward. The building up of a pressure gradient against internal resistance may be achieved gradually, because when the snail begins to move, the first waves appear at the anterior end and the pattern spreads in a posterior direction over the foot. The protraction of the posterior half of the foot through the longitudinal muscular contractions offers no difficulty. The elongation of longitudinally contracted fibres is apparently being aided in this part of the foot by the preceding contraction phase, acting partly against friction and partly against a posterior fixed point. The conditions as pictured here for the anterior and the posterior end of the animal are only possible, if at the same time an effective fixation to the ground of the central region is established. This presents no difficulty as long as the central region is occupied by an area of relaxation. When this area is being detached, it can be imagined that it is being propelled forward between the two adjacent areas of fixation in the manner diagrammatically represented in Fig. 5, *4*, while the anterior and posterior regions correspond to Fig. 5, *2* and 5, *3* respectively. These suggestions concerning the internal mechanism, however, can only be regarded as tentative, and will require further consideration.

There does not appear to exist any fundamental difficulty in imitating by means of a model the kinematic and kinetic effects of the external mechanism as observed on the sole of the snail. The musculature of the foot may be represented by a series of inflated rubber balloons attached to each other. The mechanical effect of longitudinal contraction can be achieved by deflation, and extension by inflation of

the balloon. An inflated balloon will rest firmly, with a large surface on the ground; while a deflated balloon, when situated between two inflated ones, will be lifted off the ground.

The displacement of the whole chain of balloons under the influence of successive deflation, followed by inflation of the individual balloons, spreading in postero-anterior direction over the whole series is shown in Fig. 12. To reproduce the kinetic effects of the snail's foot it would be necessary for posterior deflation in one balloon to preceed slightly the inflation of the one behind, e.g. the change from position C to position D should be made so that the process of deflating balloon 4 is somewhat in advance of the inflation of balloon 5. Thereby a tension will be set up between balloons 3 and 6. If, on the other hand, in the change from position E to F, inflation of balloon 3 preceeds deflation of 2, a longitudinal thrust will exist between balloons 4 and 1. Under these conditions the maximal static reaction from the ground must be expected under the inflated balloon nearest the central point of the chain, while sliding friction under two adjacent active balloons will remain the same throughout, and independent of whether the forward motion is effected by push or by pull.

It is essential in animals like the earthworm or the snail, which creep over a solid substrate through locomotory waves of a peristaltic nature, that the regions of fixation to the ground should undergo the least possible deformations so as to ensure a good hold. If the changes of contraction and elongation are smooth and of a sinusoidal character, the turning points of muscular activity are more

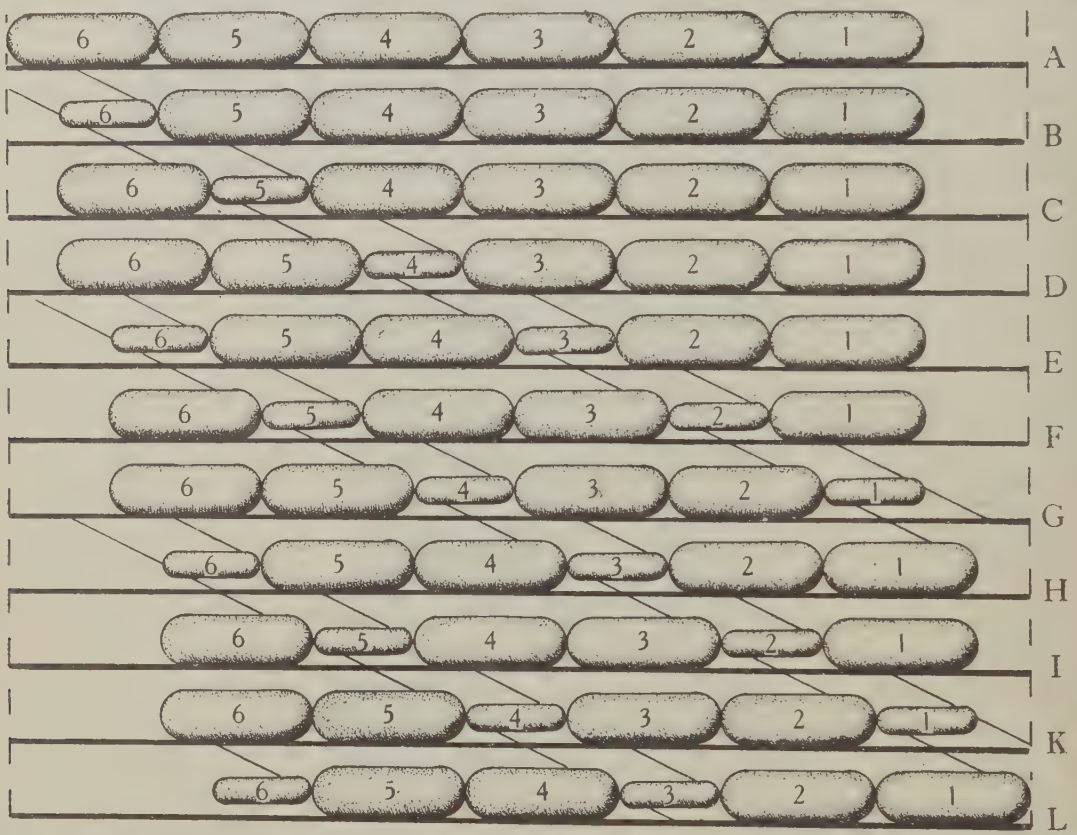


Fig. 12. Model illustrating the mechanism of locomotion in the snail. Beginning from the posterior end the balloons are successively deflated and reinflated as shown in the figure. Deflation corresponds to longitudinal contraction, inflation to elongation. The oblique lines indicate the passage of waves of activity.

suitable than any other to be used as anchors, i.e. fixation should be effected either (i) during the state of maximal longitudinal contraction, or (ii) during the state of maximal elongation. Both types of fixation involve some fundamental differences in the whole locomotory mechanism. The former is realized in the earthworm, the latter in the snail. If fixation is achieved through adhesion, its effectiveness will depend on a large and smooth surface area, and the elongated regions are more profitably used; this type of locomotion is suited for movement over smooth surfaces. It is imperative in this type of

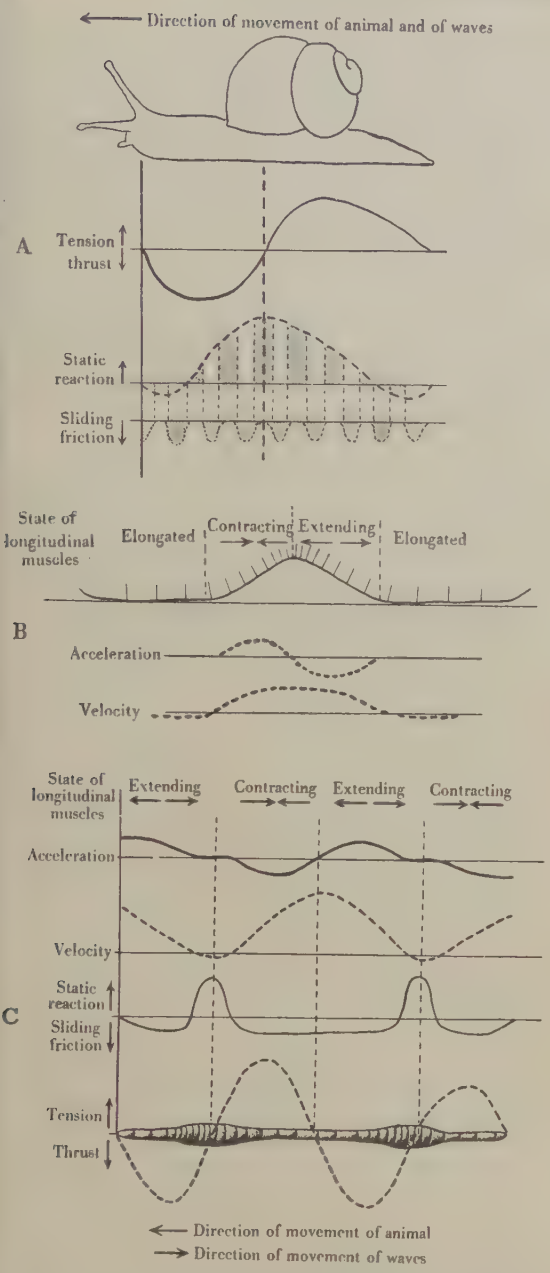


Fig. 13. Diagrammatic summary of kinematic and kinetic effects as observed on the sole of the snail in comparison with the earthworm. A. Kinetic effects of the foot as a whole; B. Kinematics of an individual locomotory wave; C. The same in the earthworm.

locomotion that acceleration of the sole should coincide with the phase of longitudinal contraction, as a result of which the locomotory waves must travel in postero-anterior direction.

In the earthworm, on the other hand, fixation through setae is restricted to the longitudinally contracted segments, and acceleration of the segments coincides with the phase of elongation, i.e. the locomotory waves move posteriorly. These differences are graphically summarized in Fig. 13 which also shows that, while in the earthworm each individual locomotory wave can be considered as an independent mechanical unit, this is not the case with the snail, because so far as the reactions from the ground are concerned, the anterior and the posterior regions represent liabilities for the central region. This may possibly bring about a more diffuse distribution of the adhering surface.

The profound bearings which such differences in the kinetics of movement must have with regard to the whole reflex life of different animals will be discussed elsewhere, but it may be indicated that if a snail encounters an exceptional resistance during progression, it will have to increase its hold on the ground, and it can do so only by relaxing longitudinal fibres on the sole, whereas the earthworm, to achieve a similar effect, must respond by longitudinal contraction. Moreover, the problem of proprioceptive control or regulation of so uniform a locomotory pattern as exhibited in the snail is of special interest, as the mechanical conditions under the anterior and the posterior regions of the foot appear reversed. Whether one is to infer from this that local reflexes operate differently at the two extremities of the snail's foot, or whether any proprioceptive activity on the pedal surface is dependent on the deformation of the muscles rather than the development of tension, only future work can decide.

SUMMARY

1. An attempt has been made to analyse the kinetic effects as observed on the sole of a gastropod in locomotion, and to illustrate these effects by analogy with mechanical models.

2. The internal mechanism involves during locomotion an internal force of longitudinal contraction, and an internal force of extension: the former is considered to be represented by the contracting longitudinal muscles, the latter is probably produced by hydrostatic pressure.

The external forces acting on the animal at any one moment represent the balance of the two antagonistic internal forces.

3. During normal ambulation in *Pomatias elegans* the posterior margin of the foot is exclusively protracted by the longitudinal contraction of the musculature of the foot; the anterior margin is propelled forward exclusively by the force of extension.

4. There is no evidence in favour of an antagonism of longitudinal and transverse fibres in *Pomatias elegans*; both sets of muscles appear to contract and relax synchronously in one half of the foot and to act antagonistically with both sets of muscles in the other half.

5. The external forces in *Pomatias* are set up between one area of fixation and an area of dynamic friction.

6. An experimental analysis of the snail showed the existence of an external force of extension (longitudinal thrust) acting between the anterior and the central region of the sole. A similar force of longitudinal contraction (tension) acts between the central and the posterior end. Both forces are of the order of 2·5 g. in *Helix pomatia*, and about 1 g. in *H. aspersa*.

7. A static reaction from the ground has been demonstrated to exist under the relaxed parts of the sole. This force reaches a maximum at a point near the central region of the foot.

8. Dynamic friction has been recorded under the forward gliding zones of contraction.

9. Static thrusts are developed between successive areas of fixation in the anterior region, while similar tensions can be observed posteriorly.

10. The foot of the snail as a whole must be considered as a mechanical unit; the individual locomotory waves do not represent mechanically balanced systems.

I wish to express my sincere thanks to Prof. J. Gray, F.R.S., for advice and for his interest in this work. I also wish to acknowledge my indebtedness to Mr D. G. Gilmour and Dr J. E. Smith for their kindness in reading the manuscript and for their friendly criticism.

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THE FERMENTATION OF CARBOHYDRATES IN THE RUMEN OF THE SHEEP

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(With Six Text-figures)

INTRODUCTION

The majority of workers are agreed that the digestion of carbohydrate in the rumen is brought about by the micro-organisms living there, and furthermore, that the microflora plays a much larger part in these processes than the microfauna, for if the Protozoa are removed by drenching with dilute copper sulphate, the digestive processes continued unhampered (see Van der Wath, 1942).

The precise role of the microflora is still a matter of controversy. One school of thought stresses the nutritional importance of the volatile fatty acids found in the rumen, the end-products of the activity of the microflora. This view is typified by the work of Phillipson (1942), Phillipson & McAnally (1942), Barcroft, McAnally & Phillipson (1944 *a*, *b*). The opposing view is that the volatile fatty acids are of little nutritional value to the animal, and that it is the microflora itself which is important, serving as a source of carbohydrate, protein and the B group of vitamins. This latter point of view is favoured by Baker who states that 'The conclusion has therefore been provisionally drawn that it is the substances synthesized such as microbial protein and polysaccharide, rather than the initial products of decomposition, such as organic acids, that are utilized by the host animal' (Baker, 1942*b*; see also Baker, 1942*a*). Baker also maintains, on the basis of direct microscopic observation of rumen contents, that the microflora converts the starch and fibre of the diet into bacterial starch. Starch is specified because a number of the rumen micro-organisms stain blue with iodine. It is assumed by Baker, though never tested experimentally, that these organisms, laden with starch, pass through the fore-stomachs into the small intestine, where the starch is hydrolysed to glucose by the pancreatic and intestinal amylases. It is equally possible that, during the time taken to pass from the rumen to the small intestine, the micro-organisms use up their store of polysaccharide by auto-fermentation, with the production of volatile fatty acids.

That the microflora do act as a source of protein is borne out by the fact that part of the protein of the diet may be replaced by simple nitrogenous derivatives such as urea, and the animal continue to thrive (Owen, Smith & Wright, 1943). The urea is used as a source of nitrogen by the microflora, the protein of which subsequently becomes available to the animal. Pearson & Smith (1943 *a*, *b*, *c*) have shown that when rumen liquor from a steer with a permanent rumen fistula is incubated *in vitro* with urea and either glucose or maltose, there is a significant increase in the amount of protein present; this is perhaps not entirely unexpected. The synthesis of the B group of vitamins in the rumen has been demonstrated by Wegner, Booth, Elvehjem & Hart (1940).

These opinions are not necessarily contradictory, and it seems certain that the ruminant utilizes both the products of the activity of the microflora—the volatile fatty acids—and the microflora itself. What is more important is the relative amounts of each available to the animal. Phillipson (1942) has also stressed this point. Owing to the difficulty of placing a permanent fistula between the abomasum and the duodenum it has so far proved impossible to estimate how much, if any, of the bacterial protein and polysaccharide passes from the fore-stomachs to the small intestine *per diem* and how much of this is available to the animal. Until this surgical problem is solved, and the measurements made, it will be impossible to assess with any degree of precision the nutritional significance of the rumen microflora to the animal.

On the other hand, such technical difficulties do not impede the study of the volatile fatty acids in the rumen, and recently it has proved possible to assess fairly accurately their importance to the animal. It has been shown that considerable quantities of volatile fatty acids are produced in the rumen of sheep after a meal (Phillipson, 1942; Phillipson & McAnally, 1942). These workers have also shown that ingestion of diets rich in soluble carbohydrate results in a rapid production of volatile fatty acids, with lactic acid as a probable intermediary. Introduction of solutions of glucose, fructose or sucrose directly into the rumen via a fistula, produces a similar rapid rise in volatile fatty acids. With diets of hay supplemented by bran and oats, or hay alone, there is a much slower rise in the concentration of volatile fatty acids, and similar

results were obtained when starch or cellulose were introduced directly into the rumen. Quin (1943), also working with sheep, has provided complementary evidence, obtained both from *in vivo* and *in vitro* studies. He did not follow the production of volatile fatty acids, but measured the rate of gas production in the rumen after feeding a given diet, or introducing a pure carbohydrate directly into the rumen.

Phillipson & McAnally (1942) showed that the concentration of volatile fatty acids is high in the rumen and low in the abomasum, and they put forward the suggestion that these compounds are absorbed from the rumen, reticulum and omasum. Barcroft *et al.* (1944*a*) have shown that, in the sheep, there is a high concentration of volatile fatty acids in the blood flowing from the rumen, reticulum, and omasum, whilst in the blood from the abomasum, small intestine and systemic circulation the amounts of volatile fatty acid are negligible. The blood from the caecal veins also contains significant amounts, and this is in agreement with the observation that the intestinal contents are subjected to a second fermentation in the caecum. Direct measurement of the rate of absorption of volatile fatty acids from the rumen and reticulum alone indicates that a significant proportion of the animal's calorific requirements could be obtained from these compounds if they be so used, apart from the amounts absorbed from the omasum and caecum.

The nature of the volatile fatty acids present in the rumen is not known with any degree of certainty. Mangold (1929, p. 326) states that the following acids have been observed: formic, acetic, propionic, *n*-butyric, *iso*butyric and valeric. Barcroft *et al.* (1944*b*), on the basis of the rate of steam distillation of the volatile fatty acids obtained from the rumen of sheep, considered that acetic acid was the principal component, and was associated with small amounts of higher fatty acids; they obtained no evidence for the presence of formic acid.

In the same way very little is known of the organisms responsible for the fermentation of carbohydrates in the rumen. Van der Wath (1942) observed that starch was digested by a coccus which simultaneously stained blue with iodine; it also fermented glucose with a similar result. This organism was isolated in pure culture and was found to ferment both glucose and starch with the production of undetermined acidic products. Quin (1943) observed a similar organism in the rumen of sheep and, if the diet was rich in soluble carbohydrate, a yeast-like organism. The latter fermented glucose rapidly both *in vivo* and *in vitro* and at the same time formed a substance which caused it to stain brown with iodine. As it multiplied by binary fission Quin classified it as a Schizosaccharomycete, and named it, provisionally, *Schizosaccharomyces ovis*. It was not isolated in pure culture, nor were its fermentation products identified. A similar organism was found by McDougall (private communication) to develop in the rumen of sheep when a diet of hay was supplemented by mangolds.

Pochon (1934, 1935) claims to have isolated in pure culture a cellulose-fermenting organism from ox rumen contents. The products formed from cellulose were ethanol, and formic and acetic acids, and later in the fermentation a little propionic acid. It is emphasized that formic was supposed to account for some 75% of the total volatile fatty acids produced and some 64% of the cellulose used. The production of such a mixture of compounds by *one* organism from *one* substrate has no parallel in bacterial fermentation, and confirmation of such a unique claim is clearly desirable.

From this brief review of the volatile fatty acids in the rumen and the agents responsible for their production it is clear that there are many gaps in our knowledge. The present work deals with (1) the nature of the volatile fatty acids present in the rumen; (2) the nature of the bacteria producing them; (3) the effect of diet on the flora of the rumen.

METHODS

Rumen contents. The rumen contents used in these experiments were either taken direct from sheep with permanent rumen fistulae, or were obtained from the animal immediately after slaughter at the abattoir; in the latter case, the samples were transported to the laboratory in a thermos flask. The crude rumen contents were filtered through muslin and the liquor so obtained used both for analysis and for the inocula in the *in vitro* experiments.

The in vitro *fermentation.* While the *in vitro* approach provides a much greater degree of control over the experiment, it is necessary so to adjust conditions that there is the least possible divergence from those which hold *in vivo*. An outstanding feature of the rumen is the large inflow of saliva from the parotid glands, amounting, in the case of the sheep, to some 3 l./24 hr. (McDougall, private communication). This forms the basal medium in which the rumen organisms grow, the substrate being provided by the diet. The saliva is approximately 0·1 *N* in respect to bicarbonate and contains, in addition, some 30–80 mg. phosphate P/100 ml. (McDougall, private communication). The bicarbonate is the main buffer, and the *p*H of the rumen contents is maintained around neutrality. The gas phase consists largely of CO_2 with up to 30% methane, oxygen rarely exceeds 1% (Mangold, 1929, p. 148). The fermentation is thus almost anaerobic. The following inorganic medium was chosen as approximating to the conditions in the rumen:

0·2 *M* $NaHCO_3$	100 ml.
0·154 *M* KCl	4 ml.
0·11 *M* $CaCl_2$	3 ml.
0·154 *M* KH_2PO_4	1 ml.
0·154 *M* $MgSO_4.7H_2O$	1 ml.
0·156 *M* $(NH_4)_2PO_4$	5 ml.

The medium was made up from stock solutions as required and saturated with pure CO_2 immediately after preparation. The fermentation vessel was a 150 ml. Buchner flask fitted with a rubber bung carrying a gas inlet tube closed by a stopcock; the tube was adjusted so as to dip below the surface of the medium; and the side arm of the flask was fitted with a Bunsen valve. 40 ml. of the medium were measured into the flask, and to this was added either 5 ml. of a 10% solution of the substrate, or, where an insoluble substrate was used, 5 ml. of distilled water. The inoculum was 5 ml. of rumen liquor, making a total volume of 50 ml. After inoculation the flask was stoppered and gassed thoroughly with CO_2 and incubated at 38°.

The in vivo *fermentation.* The *in vivo* fermentation of glucose was studied on two Hampshire Down sheep, aged about 1 year, fitted with permanent rumen fistulae. The animals were fed on the morning of the day preceding the experiment, and food was then withheld until the experiment finished 48 hr. later, access to water being allowed at all times. 24 hr. after the last meal a sample was withdrawn from the rumen, via the fistula, for analysis. A second sample was withdrawn an hour later and immediately following this a dose of glucose (100 g. dissolved in 400 ml. distilled water at 38°) was introduced, again through the fistula. Further samples of rumen contents were then taken at suitable intervals during the next 24 hr. to study the fate of the added glucose.

The substrates. Lactic acid (B.D.H. Analar), adjusted to *p*H 7 with NaOH, and glucose (B.D.H. Analar) were used. The cellulose was prepared from Whatman no. 1 filter paper. It was first thoroughly macerated in boiling distilled water, filtered off at the pump, and washed with a large volume of distilled water followed by 95% ethanol; it was then dried in a steam oven. The washing with ethanol was essential, as it resulted in a light fluffy product which disintegrated readily on shaking with water. If this step was omitted, or if an insufficient volume of ethanol was used, the product dried in hard lumps which would not disintegrate in water. The dried grass used was kindly supplied by the Hannah Dairy Research Institute, to whom the author wishes to extend his grateful thanks.

Methods of chemical analysis

(1) *Volatile fatty acids.* These were determined by the silica gel partition chromatogram (Elsden, 1945). By this method the acids were identified, separated quantitatively one from the other, and the fractions titrated with *ca.* 0·01 *N* NaOH. The volatile fatty acids were separated from the non-volatile acids by the distillation procedure of Friedemann (1938).

(2) *Glucose.* The method of Hanes (1929) was used. Solutions were deproteinized by the addition of $\frac{1}{5}$ vol. of saturated lead acetate, and the excess lead removed by solid Na_2HPO_4; after filtration a suitable aliquot was taken for analysis. Experience showed that the more usual methods of protein precipitation could not be used because of the large and variable amounts of bicarbonate encountered. The procedure adopted was found to be adequate and entailed no loss of glucose.

(3) *Lactic acid.* This was determined on a copper-lime filtrate of rumen liquor, without a preliminary removal of protein, by the method of Friedemann & Kendall (1929). 5 ml. of the rumen liquor were pipetted into a 50 ml. volumetric flask; 5 ml. of 20% $CuSO_4.5H_2O$ (w/v) were then added, followed in turn by distilled water, 5 ml. 10% suspension of $Ca(OH)_2$ and sufficient distilled water to make up to the mark. The mixture was allowed to stand for 30 min., filtered, and a suitable aliquot taken for analysis.

(4) *Ethanol.* The method of Friedemann & Klaas (1936) was used without modification.

(5) *Higher alcohols.* These were tested for by oxidizing with bichromate according to Friedemann (1938), and the fatty acids produced identified on the partition chromatogram. No higher alcohols were in fact observed.

RESULTS

The volatile fatty acids in the rumen. Rumen contents from a number of sheep and an ox were analysed for volatile fatty acids. The results (Table 1) are expressed as mM. fatty acid per 100 ml. rumen liquor. Three acids were invariably present: acetic, propionic and a butyric isomer (the method does not

Table 1. *Volatile fatty acids in the rumen of the sheep and ox*

The results are expressed as mM. volatile fatty acid/100 ml. rumen liquor. * indicates that the animal was at grass, and † that the animal was fitted with a permanent rumen fistula.

Animal	Acetic	Propionic	Butyric	Total
Sheep F†	5·45	1·18	0·78	7·41
,, F†	3·28	1·2	0·84	5·32
,, F*†	8·1	3·68	2·14	13·92
,, C	1·34	0·86	1·6	3·8
,, D*†	7·68	2·14	1·3	11·12
,, E	7·6	2·2	2·44	12·24
,, G	2·52	0·7	0·7	3·92
,, W.F.†	1·51	0·48	0·26	2·25
,, W.F.†	3·04	0·71	0·69	4·44
,, 1*	7·78	2·46	1·46	11·7
,, 2*	5·25	1·52	1·48	8·25
,, 3*	5·42	1·52	1·16	8·1
,, 4*	8·2	1·82	1·38	11·4
,, 5*	11·35	4·05	2·6	17·8
,, 6*	7·73	2·23	2·14	12·1
,, 7*	7·9	1·96	1·67	11·53
,, 8*	8·52	2·18	2·1	12·8
,, 9*	7·0	2·26	1·94	11·2
Ox 1	4·48	0·92	0·62	6·02

distinguish between isomers). In addition, a very fast-moving band, corresponding to a valeric acid, was usually observed on the chromatogram, but, as the amount was small, the practice was adopted of collecting this along with the butyric fraction and titrating as one. The figure for butyric acid is thus a composite one. It will be observed that there was considerable variation from one animal to another and even in the same animal; nevertheless, the following conclusions may be drawn. With the exception of one animal, C, acetic acid predominated and amounted to 55–75% of the total; propionic acid was next, followed by the butyric-valeric fraction. The distillation method adopted for the preliminary separation of the volatile fatty acids destroyed formic acid; but even when the oxidizing agent ($HgSO_4$) was omitted, no formic was found. It is of interest that the same fatty acids, in more or less the same proportions, were found in the rumen liquors of the ox, and it may well be that such a mixture is characteristic of all ruminants, though much more work would have to be carried out before any such generalization can be safely made.

The fermentation of cellulose. Preliminary experiments *in vitro* under the conditions described above indicated that cellulose was rapidly fermented with the production of volatile fatty acids and considerable quantities of gas. About 1 ml. of 0·01 *N* total acid was produced per mg. cellulose fermented, and in consequence a considerable quantity of the gas formed must be CO_2 arising from action of the acids on the bicarbonate buffer. No detailed investigation has been made of the composition of the gas. Most of the ammonia N of the medium had disappeared by the end of the fermentation.

Table 2 records the detailed analysis of the volatile fatty acids produced by the *in vitro* fermentation of cellulose. The fermentations were not taken to completion nor, in this series of experiments, was the residual cellulose estimated, so that carbon balance sheets cannot be constructed. The results were surprisingly uniform, and under the conditions used propionic and acetic acids were the chief volatile fatty acids produced. The position of butyric acid is uncertain, for the amounts produced over and above the control were so small as to be almost within the limits of the analytical procedure. Propionic acid predominated. This came as a surprise, and its presence was further confirmed in two ways. First by the microchemical test of Musicant & Kaszuba (1939), which was subsequently used as a routine test throughout, and secondly by the isolation of the silver salts of both propionic and acetic acids. The procedure for the latter was as follows.

The mixed volatile fatty acids from 40 ml. of fluid from a cellulose fermentation were separated by the usual distillation procedure and taken up in 50 ml. chloroform as for the chromatographic method, and the chloroform extract fractionated on the partition chromatogram, using ten columns in all. The acetic and propionic fractions were collected separately and titrated with 0·03 *N* NaOH. This gave solutions containing 76·3 and 55 mg. propionic and acetic acids respectively as their sodium salts. Both were treated in the following way. The solution was made up to 200 ml. and acidified with 10 ml. of 2 *N* H_2SO_4 containing 10% (w/v) $HgSO_4$, and 50 g. of $MgSO_4 \cdot 7H_2O$ added. The volatile fatty acid was distilled off, heating being continued until the $MgSO_4$ commenced to crystallize. The distillate was shaken up with excess solid Ag_2CO_3, and when the acid had been neutralized the residual solid was filtered off. The solution containing the silver salt of the fatty acid was evaporated down to incipient crystallization and then left in the refrigerator. Next day the product was filtered off and recrystallized from boiling water. The crystals were filtered off and dried in a vacuum desiccator over H_2SO_4. The mother liquors were worked up and a second crop was obtained. In all 96·3 mg. of silver propionate, and 49·1 mg. silver acetate were recovered; the theoretical yields, on the basis of the initial amounts of each acid, were 189 and 153 mg.

Table 2. *Volatile fatty acids, in mM., produced during the* in vitro *fermentation of cellulose by rumen contents from the sheep*

Figures in parentheses refer to the volatile fatty acids in the control experiment incubated without cellulose. XS implies that an excess of cellulose, > 1 g., was used. Those sheep marked † were fitted with a permanent rumen fistula.

Exp.	Sheep	Cellulose mg.	Total volatile acids	Acetic	Propionic	Butyric	Time hr.
1	F†	500	4·59 (0·53)	2·38 (0·37)	2·08 (0·1)	0·13 (0·06)	95½
2	F†	500	3·17 (0·38)	1·07 (0·22)	1·98 (0·07)	0·12 (0·09)	88¾
3	F†	500	3·91 (0·60)	1·79 (0·34)	2·04 (0·19)	0·09 (0·07)	88
4	A†	530	4·50 (0·69)	1·95 (0·41)	2·33 (0·18)	0·2 (0·1)	90
5	B	XS	6·75 (0·91)	3·04 (0·53)	3·23 (0·16)	0·48 (0·22)	81½
6	D†	505	4·78 (0·0)	2·05 (—)	2·56 (—)	0·17 (—)	112
7	E	636	5·57 (0·0)	2·30 (—)	2·81 (—)	0·46 (—)	95
8	G	XS	5·91 (0·0)	2·52 (—)	2·96 (—)	0·43 (—)	141½
9	F†	XS	5·06 (0·19)	2·14 (0·10)	2·78 (0·06)	0·14 (0·03)	95½

respectively. The compounds analysed as follows. (Found: C, 20·2; H, 3·1; $C_3H_5O_2Ag$ requires C, 19·9; H, 2·8. C, 14·8; H, 1·8; $C_2H_3O_2Ag$ requires C, 14·4; H, 1·8.)

The fermentation of glucose. Table 3 gives the detailed analysis of the volatile fatty acids produced by the *in vitro* fermentation of glucose. Acetic, propionic and butyric acids were formed. Propionic acid predominated as in the case of cellulose but, in contrast to the latter, significant amounts of butyric acid were formed. Phillipson & McAnally (1942) showed that when glucose was fermented in the rumen there was a transient appearance of lactic acid, and in view of this finding the course of the *in vitro* fermentation of glucose was followed in more detail.

Table 3. *Votatile fatty acids produced during the* in vitro *fermentation of glucose by rumen contents from the sheep.* 500 *mg.* (2·78 *mM.*) *were fermented in each experiment*

Figures in parentheses refer to the volatile fatty acids in the control experiment incubated without glucose. The sheep marked † were fitted with a permanent rumen fistula.

Exp.	Sheep	Total volatile acids	Acetic	Propionic	Butyric	Time hr.
1	F†	4·54 (0·60)	1·56 (0·34)	2·22 (0·19)	0·72 (0·07)	88
2	A†	3·25 (0·69)	1·02 (0·41)	1·83 (0·18)	0·40 (0·10)	90
3	C	3·30 (0·0)	0·57 (—)	1·50 (—)	1·23 (—)	88½
4	B	4·41 (0·91)	1·41 (0·53)	1·93 (0·16)	1·07 (0·22)	81½
5	D†	4·80 (0·0)	1·50 (—)	2·36 (—)	0·94 (—)	112
6	E	3·04 (0·0)	0·91 (—)	1·18 (—)	0·95 (—)	95
7	G	2·79 (0·0)	0·39 (—)	1·30 (—)	1·10 (—)	141½

The fermentations were conducted on ten times the usual scale mentioned above. Immediately following the addition of glucose a sample was removed from the mixture for analysis, and the remainder gassed

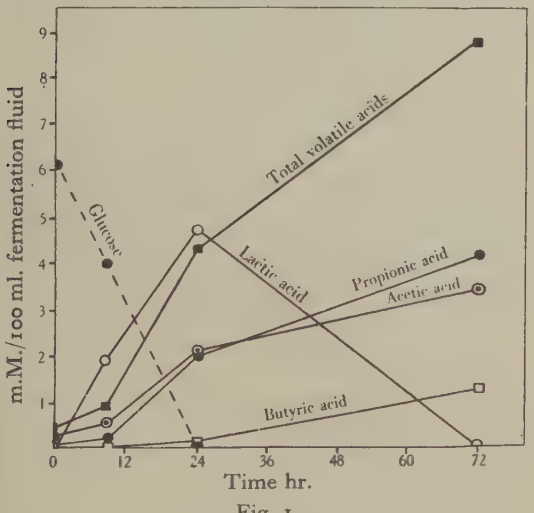

Fig. 1.

with CO_2 and placed in the incubator at 38°. At suitable intervals of time samples were taken and the main solution re-gassed with CO_2 prior to replacing in the incubator. The results are expressed graphically in Fig. 1. It will be noted that the glucose was used up within 24 hr. and that large quantities of lactic acid and volatile fatty acid, chiefly propionic and acetic, were then found to be present. During the next 48 hr. the lactic acid disappeared, and there was a corresponding increase in the volatile fatty acids. Propionic and acetic acids were the main products, but it is important to notice that some butyric acid was also produced.

Comparison of the fatty acids of the rumen liquor with those produced from the *in vitro* fermentation of both cellulose and glucose reveals a qualitative similarity; quantitatively, however, there is a discrepancy. Thus, acetic acid predominated *in vivo*, whereas propionic acid was the main product *in vitro*.

It became necessary, therefore, to study further the *in vivo* fermentation of carbohydrate, and in particular the nature and amounts of the volatile fatty acids produced.

For this purpose two Hampshire Down sheep, W.F. and B.F., with permanent rumen fistulae, were selected; B.F. was used mainly to confirm the observations made with W.F. At the time this series of experiments were initiated W.F. was on a diet of poor-quality hay and was not putting on weight, though the diet appeared to be adequate calorifically. Reference has already been made to the general method of conducting the experiments. The results of the first experiment are given in Fig. 2. There was a very slow utilization of glucose, so that 9 hr. after dosing there was still a significant amount remaining in the rumen liquor. This slow rate of glucose fermentation was also reflected by the almost insignificant increase in lactic acid and total volatile fatty acids; at no stage was a peak observed. This was in contrast to the experiments of Phillipson & McAnally (1942). The experiment was repeated at intervals over a period of 2 months with similar results.

By this time the animal was in such poor condition that the diet was changed to one consisting of good-quality clover hay, and after 14 days on this diet the experiment was repeated. As will be seen from Fig. 3 a considerable change had occurred. The animal was now able to ferment glucose rapidly, and

the bulk of the glucose had disappeared within 6 hr. of dosing; there was also a significant increase in volatile fatty acids, and lactic acid made its appearance in quantity. Parallel to the chemical analyses, obser-

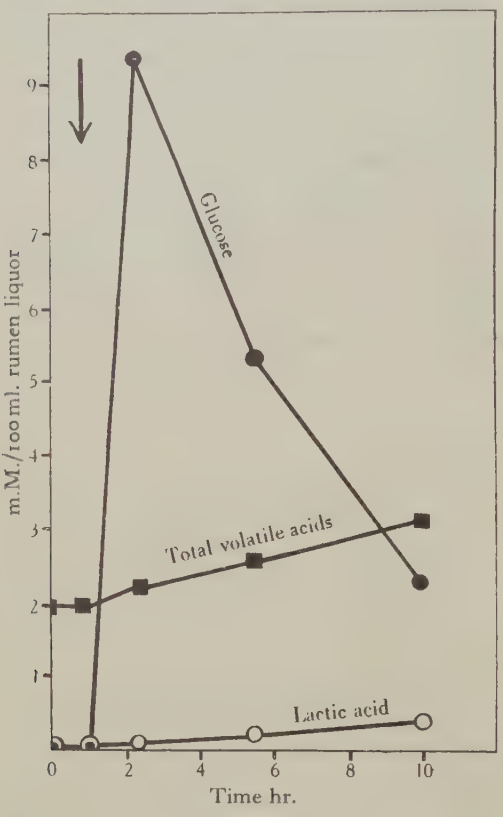

Fig. 2.

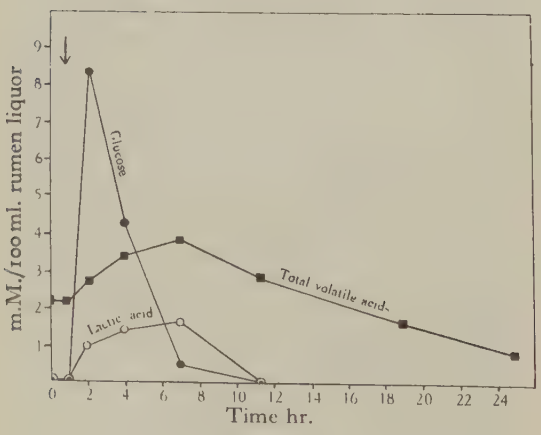

Fig. 3.

vations were made on the micro-organisms throughout the experimental period. For this purpose 5 ml. of rumen liquor were taken and mixed with 1 ml. of 50% (v/v) formalin as a preservative; the sample could then be examined at leisure. Prior to the microscopic examination, 1 ml. of double strength Gram's iodine was added in order to reveal the presence of iodophile organisms. The samples taken prior to the introduction of the glucose were pale brown in colour, and microscopic examination, whilst revealing the presence of Protozoa and numerous bacteria, amongst which could be seen large diplococci and streptococci, showed no preferential staining with iodine. On the other hand, samples taken 1 hr. after dosing with glucose and treated with iodine were a brownish purple in colour, and under the microscope could be seen many of the large diplococci and streptococci now stained a deep blue with the iodine. Similar organisms were observed attached to pieces of plant debris, and it is clear that the presence of iodophile organisms does not necessarily imply that the polysaccharide store, revealed by the iodine, was produced from the material to which the organism was attached. At this stage the Protozoa were filled with iodophile cocci: this has also been observed by Van der Wath (1942).

No attempt was made to count the numbers of iodophile cocci owing to their uneven distribution in the fluid. Qualitatively, it may be said that the staining reaction remained constant as long as glucose persisted in the rumen liquor, and thereafter diminished steadily, so that by the 18th hour it had disappeared; but organisms, similar in size and shape to those which had given the reaction, were to be seen in plenty. The sheep B.F., which had been maintained on the same poor-quality hay, but supplemented with bran and crushed oats, showed a similar series of changes to those recorded in Fig. 3, and the microflora showed an intense iodine reaction within 30 min. of introducing the glucose. The same types of micro-organisms, as far as could be judged by microscopic examination alone, were present.

Forty-two days after changing the diet of W.F., the animal's response to glucose was again tested. The results are given in Fig. 4. Maximum concentration of lactic acid was reached 1 hr. after dosing, earlier than in the previous experiment, and it had entirely disappeared by the 6th hour. Glucose was used much more rapidly than before, and had been completely utilized by the 2nd hour. There was a large increase in the concentration of total volatile fatty acids, reaching a peak 3 hr. after dosing.

Fig. 5 shows the changes occurring in the individual volatile fatty acids during this experiment. The important feature is the increase in propionic acid as compared with acetic acid; this point is further emphasized by Table 4, in which the ratio of acetic to propionic acid is given for the various stages in the fermentation. Some butyric acid was also produced.

As in the previous experiment, rumen liquor drawn prior to the administration of glucose showed no marked coloration on treating with iodine, but subsequent to the dosing it was coloured a dark

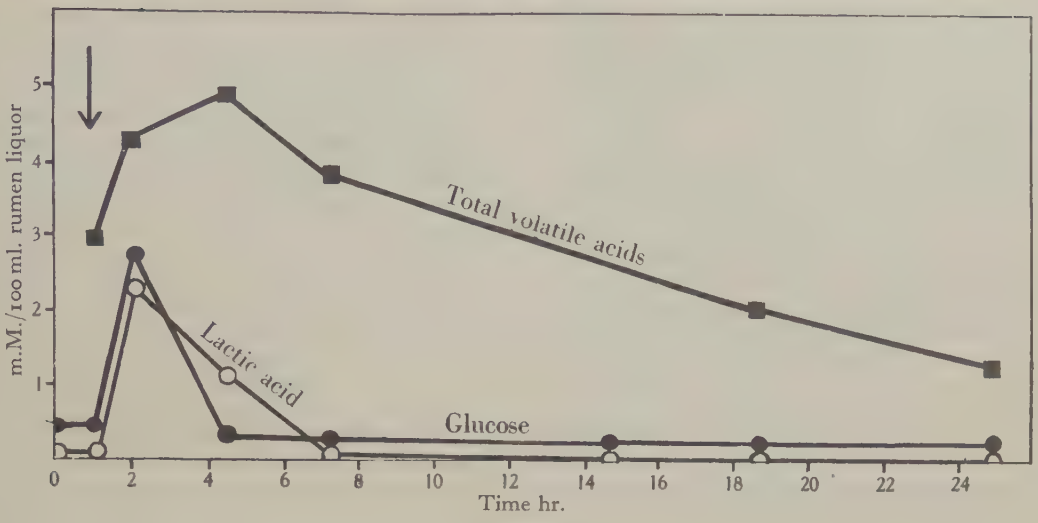

Fig. 4.

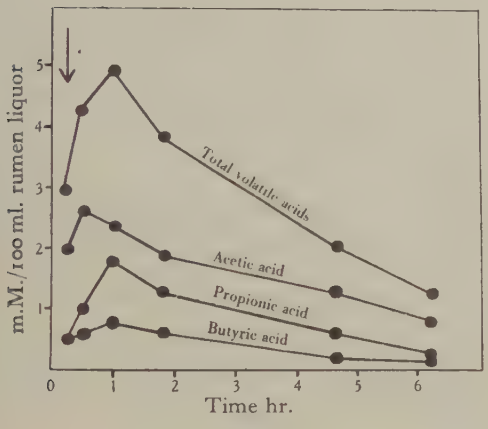

Fig. 5.

Table 4. *The concentration of acetic and propionic acids, and the ratio of acetic to propionic in the rumen liquor of sheep W.F. at various times during the fermentation of glucose. The values recorded are expressed as mM. acid/100 ml. rumen liquor*

Time hr.	Acetic	Propionic	Acetic/ propionic
0	2·01	0·47	4·28
1	2·68	1·01	2·66
3	2·34	1·79	1·3
6·25	1·91	1·32	1·45
17·5	1·27	0·58	2·19
24	0·80	0·25	3·2

brown. This reaction persisted up to the 6th hour and then steadily diminished so that by the 18th hour it had disappeared. Microscopic examination revealed that a profound change had occurred in the rumen flora. Even before the addition of glucose, numerous large, oval, colourless, yeast-like organisms could be seen, similar to the schizosaccharomycete described by Quin (1943). Subsequent to the addition of glucose, these organisms stained a deep brown with iodine, and many were observed to be in a state of active multiplication by binary fission. There were very few iodophile cocci present, and it appeared as

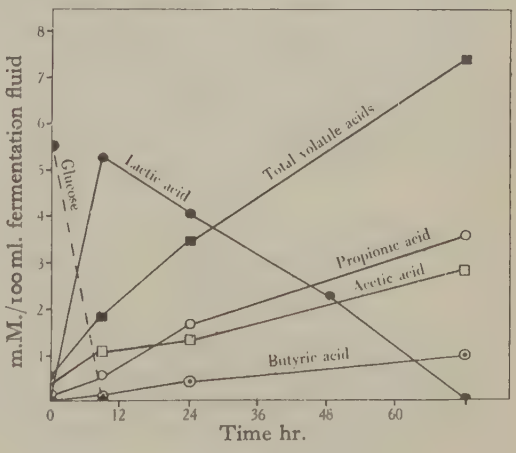

Fig. 6.

though they had been supplanted by the yeast-like organism.

With sheep W.F. it was possible to compare the *in vivo* fermentation of glucose with the *in vitro*, and to this end an experiment, similar to that recorded in Fig. 1, was performed. The results are given in Fig. 6. The *in vitro* experiment reflects, on the whole, the *in vivo*. Both resulted in the production of acetic, propionic and butyric acids, with propionic acid predominating; and in both lactic acid appeared as an

intermediary. The fact that there were considerable quantities of propionic acid appearing in each type of experiment is of importance, and will be commented on later when the origin of propionic acid is discussed.

The similarity of the chemical changes occurring both *in vivo* and *in vitro* was paralleled by the similarity in the microflora which developed. *In vitro* there were very few iodophile occi, but on the other hand large numbers of the schizosaccharomycete developed, and these stained a dark brown with iodine during the first 24 hr. of the experiment. Subsequently this ability was lost, presumably due to the removal of the polysaccharide store by autofermentation. No ethanol was detected at any stage of the experiment, and thus, if the organism produced ethanol, it must have been removed as fast as it was formed. The similarity existing between the flora developing *in vitro* and that occurring *in vivo* is a further indication of the physiological nature of the *in vitro* conditions.

The fermentation of lactic acid. The experiments on the fermentation of glucose described in the previous section showed that lactic acid was produced and that it subsequently gave place to volatile fatty acids. A more detailed study of the lactic acid fermentation was made using the *in vitro* method. The results are given in Table 5. Acetic, propionic and butyric acids were produced, and, as in the case of the other substrates studied, propionic acid predominated.

The origin of acetic acid. It will have been noted that propionic acid was the major component of the volatile fatty acids produced *in vitro* from glucose, cellulose and lactic acid; whereas *in vivo*, on the normal mixed diet, acetic acid predominated. The question arose, therefore, was this discrepancy due to the fact that, *in vivo*, a very mixed substrate was fermented, and the nature of the substrate conditioned the nature of the end-products? For it is well known that, even in pure culture studies, the proportions of the end-products vary with the type of substrate fermented. Or were the fatty acids produced *in vitro* an artefact in the sense that the *in vitro* conditions were such as to favour the development of a completely different flora from that *in vivo*, with the result that different end-products were produced?

The studies on the fermentation of glucose recorded above are evidence in favour of the view that the conditions were physiological, and therefore any differences between the *in vitro* and *in vivo* fermentations were due primarily to the nature of the substrates fermented. To test this further a series of experiments was performed in which dried grass was fermented *in vitro*. Table 6 records the results obtained. It will be seen that large amounts of volatile fatty acid were produced consisting of acetic, propionic and butyric acids; acetic acid was the major constituent. These experiments, therefore, support the hypothesis that the predominance, *in vivo*, of acetic acid is due to the nature of the substrate. At

Table 5. *Volatile fatty acids, in mM., produced during the* in vitro *fermentation of lactic acid, added as sodium lactate, by rumen contents of the sheep.* 500 *mg.* (5·55 *mM.*) *lactic acid were used in each experiment*

Figures in parentheses refer to the volatile fatty acids in the control experiment incubated without lactic acid. Those sheep marked † were fitted with a permanent rumen fistula.

Exp.	Sheep	Total volatile acids	Acetic	Propionic	Butyric	Time hr.
1	F†	5·20 (0·60)	2·10 (0·34)	2·76 (0·19)	0·34 (0·70)	88
2	A†	3·38 (0·69)	0·73 (0·41)	2·37 (0·18)	0·28 (0·10)	90
3	C	3·53 (0·0)	1·36 (—)	2·02 (—)	0·15 (—)	88½
4	B	3·78 (0·91)	0·92 (0·53)	1·37 (0·16)	1·49 (0·22)	81½
5	G	2·95 (0·0)	0·28 (—)	1·52 (—)	1·15 (—)	141½

Table 6. *Volatile fatty acids, in mM., produced during the* in vitro *fermentation of dried grass by rumen contents from the sheep. An excess of dried grass was used and at the termination of the experiment there was a considerable residue*

The figures in parentheses refer to the volatile fatty acids in the control experiment incubated without grass. Those sheep marked † were fitted with a permanent rumen fistula.

Exp.	Sheep	Total volatile acids	Acetic	Propionic	Butyric	Time hr.
1	C	5·05 (0·0)	2·22 (—)	1·95 (—)	0·88 (—)	88½
2	D†	9·06 (0·0)	4·74 (—)	3·0 (—)	1·32 (—)	112
3	E	9·78 (0·0)	5·72 (—)	2·64 (—)	1·42 (—)	95
4	G	7·45 (0·0)	4·08 (—)	2·54 (—)	0·83 (—)	141½
5	F†	9·65 (0·19)	5·08 (0·10)	3·72 (0·06)	0·85 (0·03)	95½

the same time it is realized that a much more detailed study must be made before this is firmly established. Such a study requires, in the first place, a much more detailed knowledge of the carbohydrate components of animal feeding stuffs than at present exists, particularly of the so-called hemicelluloses and pentosans; and in the second place, a plentiful supply of such compounds in a relatively pure form, with which to study their behaviour both *in vivo* and *in vitro*.

The origin of propionic acid. The observation that propionic acid was normally present in the rumen, and that it was produced during the *in vitro* fermentation of cellulose, glucose and lactic acid, particularly the last-mentioned, suggested at once that members of the genus *Propionibacterium* were playing a part in the fermentations in the rumen. Attempts were therefore made to isolate these organisms both from rumen contents and from the *in vitro* fermentations.

The procedure was as follows. Tubes of Stephenson's inorganic medium, *p*H 7·4, and containing in addition 0·4% Difco yeast extract (w/v) and 1% (w/v) sodium lactate, were inoculated with a drop of the liquid under examination. Incubation was at 38° for 10 days under an atmosphere of N_2 containing 5% CO_2. At the end of this time there was a heavy growth at the bottom of the tubes. Two or three subcultures were made on the liquid medium, and finally the cultures were plated out on the same medium solidified with 2% agar. After replating a number of times pure cultures were obtained of what were considered to be members of the genus *Propionibacterium*. They had the following properties: small, Gram-positive cocci, sometimes in pairs; catalase-positive; colonies 1–2 mm. diameter, dome-shaped and cream-coloured; anaerobes, with good growth at the bottom of yeast extract, lactate agar stabs, but with no surface growth; fermented lactic acid and glucose with the production of propionic and acetic acids and CO_2. These properties are consistent with the view that these organisms are members of the genus *Propionibacterium*. In all pure cultures were isolated from the rumen contents of three sheep and an ox, and from the *in vitro* fermentations of cellulose, glucose, lactic acid and dried grass by the rumen liquor from four other sheep. The isolation of these organisms makes it highly probable that they are responsible for the production of the propionic acid found in the rumen, and that part of the acetic acid must also be ascribed to them.

DISCUSSION

As stated in the Introduction, the present communication deals with three different problems: (1) the nature of the volatile fatty acids in the rumen and the quantities of each present; (2) the organisms responsible, either directly or indirectly, for the production of volatile fatty acids and the compounds from which they are produced; (3) the effect of diet on the composition of the rumen microflora. A complete answer has been obtained to problem (1), and a partial answer has been obtained to problems (2) and (3).

Acetic, propionic and butyric acids were invariably found in the rumen, and frequently a higher acid, probably a valeric isomer. The question of formic is still undecided. It could not be found on the occasions it was sought for, but a failure to find it on a few occasions does not imply that it is never present. It must also be pointed out that the methods available for the detection and estimation of this compound are unreliable, due to a lack of specificity. To settle this point new analytical procedures are needed, and the two most promising appear to be (1) the utilization of formic hydrogenlyase, an enzyme found in *E. coli* and certain other members of the enterobacteriaceae which splits formic acid quantitatively into H_2 and CO_2; this method has already been used by Woods (1936). (2) The method for the separation of the volatile fatty acids, introduced by Schicktanz, Steele & Blaisdell (1940), based on the fractional distillation of the azeotropes of volatile fatty acids and aryl hydrocarbons.

Of the vast numbers of micro-organisms found in the rumen only three species have been definitely associated with the fermentation of carbohydrate. They are: (1) the untyped, iodophile coccus, isolated in pure culture by Van der Wath (1942), and observed but not isolated by Quin (1943) and by the author; (2) the yeast-like organism, first recorded by Quin (1943) and named by him *Schizosaccharomyces ovis*, which is also recorded in the present paper; (3) untyped members of the genus *Propionibacterium*, whose isolation from rumen contents and whose role in the breakdown of carbohydrate in the rumen are now reported for the first time.

The iodophile coccus was shown by Van der Wath (1942) to play a part in the breakdown of glucose and starch; Quin showed that it was connected with the fermentation of glucose, and similar observations are now recorded. The precise part played by this organism is still a matter of conjecture, and the products produced from these compounds are unknown; Van der Wath (1942) found that acid was produced from both, but this acid has not as yet been identified. As the organism is a coccus, it is probable that lactic acid is one of its end-products. It is certain that the organism converts part at least of its substrate into a polysaccharide resembling starch. Further study of this organism is a problem for the future.

A detailed study of the schizosaccharomycete is also required. Quin (1943) associated this organism with the very rapid fermentation that occurs when sheep are fed on a diet rich in soluble sugars. He also showed that the organism could be partially separated by fractional centrifugation, and that sus-

pensions of the organism in bicarbonate buffer fermented glucose very rapidly as judged by the rate of gas formation. More than this cannot be said at the moment, for no information was forthcoming on the nature of the other substances formed, but from the experiments recorded in the present paper and from preliminary experiments with suspensions prepared by fractional centrifugation, it seems likely that ethanol is not produced. The isolation of this organism in pure culture and a detailed study of its metabolism is a problem of immediate importance, though it is probable that useful information could be obtained from suspensions prepared from rumen liquor by fractional centrifugation.

The work described in the present paper suggests most strongly that unidentified members of the genus *Propionibacterium* are functional members of the rumen flora. The evidence can be summarized as follows: (1) members of this genus have been isolated from the rumen contents of a number of sheep and an ox; (2) lactic acid appears during both the *in vivo* and the *in vitro* fermentation of glucose, but gives place to volatile fatty acids, mainly propionic and acetic acids, though under some conditions more or less butyric acid may be formed. The ability to convert lactic acid into propionic and acetic acids is diagnostic of the genus *Propionibacterium*. This evidence makes it certain that these organisms are responsible for the formation of the propionic acid which is invariably found in the rumen. The components of the diet from which propionic acid is either directly or indirectly produced can be referred to only in general terms at this stage. The soluble sugars of the diet are sources of propionic acid; cellulose is also a possibility, for the *in vitro* experiments with this substance suggest that the propionic acid bacteria play a part in its breakdown; but it still remains to be shown that the *in vivo* fermentation of cellulose resembles the *in vitro*.

Under the conditions used the fermentation of cellulose appears to be a two-stage process, a primary breakdown of cellulose by the specific organisms, followed by a secondary fermentation of the product or products thus formed by the propionic acid bacteria. The evidence for this is that members of the genus *Propionibacterium* were isolated from the *in vitro* fermentations of cellulose, and they showed no ability to ferment cellulose. It is possible that the method of isolation was such as to cause the loss of this property; the former hypothesis seems, however, to be more reasonable. If it be accepted that the fermentation of cellulose to volatile fatty acids, a widespread phenomenon in nature, involves two or more types of organisms, then the wide variety of volatile fatty acids produced by allegedly pure cultures of cellulose fermenters is explained, for the nature of the end-products will be conditioned by the type of secondary organisms. In a similar manner the breakdown of soluble sugars in the rumen appears to be a two-stage process, the first group of organisms producing lactic acid, the second group, amongst which are the propionic acid bacteria, ferment lactic acid to volatile fatty acids.

The presence of propionibacteria in the rumen is consistent with the older work on this group. Their normal habitat was considered to be dairy produce, and the problem was to explain how they arose there. Burri (1911) demonstrated that the faeces of cows contained large numbers of propionic acid bacteria; and Thöni (1906) demonstrated their presence in natural rennet, and, what is more important, from the abomasum from which the rennet was made. This work established the fact that this group of organisms do occur in the alimentary canal of the cow, and in consequence their occurrence in the rumen is not unexpected.

The origin of acetic acid has not as yet been satisfactorily explained. Part, at least, must arise through the activities of the propionic acid bacteria, but the fact that, *in vivo*, some 55–75 % of the total volatile fatty acid of the rumen liquor is acetic acid cannot be readily explained in terms of these organisms alone. The experiments with dried grass suggest that the diet conditions the composition of the flora, which in turn conditions the nature of the end-products; and it may well be that the acetic acid arises directly from polysaccharides such as hemicelluloses and pentosans, which, as was shown by McAnally (1942), are more readily fermented in the rumen than cellulose. The protein of the diet may also give rise to some acetic acid.

The presence of propionic acid in the rumen having been discussed, it is now necessary to consider the origin of butyric acid. Like propionic acid it is produced from glucose and lactic acid; it was not formed in significant amounts from cellulose under *in vitro* conditions. The fact that it is produced from lactic acid provides a possible clue to the type of organism responsible. Recently, Barker & Haas (1944) showed that the anaerobic organisms, isolated from human intestinal contents by Lewis & Rettger (1940) and by King & Rettger (1942), fermented lactic acid to a mixture of acetic and butyric acids along with traces of a higher fatty acid, probably caproic acid. It seems possible that a similar type of organism may function in the rumen.

So far no mention has been made of the methane which is produced in the rumen. Formerly it was ascribed, without convincing evidence, to the action of the cellulose fermenters, but the recent work of Barker (1936, 1939) has demonstrated conclusively that methane is produced by the reduction of CO_2, with the simultaneous oxidation of primary or secondary alcohols, or the lower fatty acids, depending on the type of organism. The conditions in the rumen, anaerobiosis, a plentiful supply of CO_2 and volatile fatty acids, are ideal for the growth of this group of organisms. So far no search has been made for them, but, if a complete picture of the events in the rumen is to be obtained, a detailed study of this

phase will have to be made, and the classical work of Barker will be an invaluable guide.

Finally comes the question of the effect of diet on the composition of the rumen flora. Van der Wath (1942) demonstrated by direct counts that the number of bacteria in the rumen is conditioned by the composition of the diet. Thus when teff hay, consisting largely of fibre, was fed, the total count, as compared with animals on a mixed diet, was low; when this was supplemented by protein or urea, the number of micro-organisms was increased—thus N was one of the limiting factors. When the diet was further supplemented by starch or molasses, the count was again increased, and when a third supplement was given in the form of bone meal or inorganic phosphate, the total bacterial count was again raised. The technique of total counts, whilst providing useful information, is limited in its scope; thus little information of the number of species present, or the numbers of each species is obtained, for unless an organism possesses a highly characteristic shape or specific staining reaction, it is impossible to distinguish one species from another. The method of viable counts is also of little use here, and it is clear that a completely new approach is needed.

Quin (1943) established that the composition of the rumen microflora is far from constant, and is conditioned by the nature of the diet. He employed what may be termed the biochemical method of assaying the flora. He studied the *in vivo* and the *in vitro* fermentation of glucose by the rumen liquor from sheep on different diets; the rate of gas formation was taken as an index of the rate of glucose fermentation. Sheep fed on veld grass hay, a diet of low nutritional value, fermented glucose very slowly; whereas, when the diet was changed to lucerne, either fresh or as hay, the rate of fermentation was speeded up. Coincident with the change in diet was a change in the composition of the microflora. First, the iodophile coccus established itself as a dominant organism only to be succeeded by the schizosaccharomycete. This latter organism was associated with the most rapid type of fermentation. These observations have been fully confirmed by the present work, though, instead of following the rate of gas evolution, the rate of glucose fermentation was measured directly, and the products formed determined.

It is clear from all this work that a diet deficient in soluble sugars or starch does not favour the development of a flora capable of using them; it would be curious were it otherwise: and therefore when glucose is introduced into the rumen of an animal, fed on such a diet, it is fermented very slowly. From the work of Van der Wath (1942) it is evident that a starch-rich diet produces a rumen flora in which the iodophile coccus is a dominant organism, whereas a diet rich in soluble sugars, whilst resulting in a temporary dominance of the iodophile cocci, ultimately leads to the schizosaccharomycete establishing itself, accompanied by the almost complete disappearance of the cocci.

The importance of further studies of the factors controlling the composition of the microflora of the rumen cannot be over-emphasized, for it is certain that a vigorous microflora is one of the conditions essential for the health of the animal. There are also broader issues, for such studies open the way to the investigation of bacterial ecology, a field which has been as yet poorly developed.

SUMMARY

1. Acetic, propionic and butyric acids are the main volatile fatty acids in the rumen of the sheep. Acetic acid accounts for 55–75% of the total.

2. Cellulose, glucose and lactic acid are rapidly fermented *in vitro* by rumen contents with the production of acetic, propionic and butyric acids. Propionic acid is the major component in all cases: very little if any butyric acid is formed from cellulose.

3. The *in vitro* fermentation of glucose closely resembles the *in vivo*.

4. The *in vitro* fermentation of dried grass yields the same three acids, but with acetic acid predominating.

5. Members of the genus *Propionibacterium* have been isolated from the rumen, and evidence is presented to show that these organisms are responsible for the production of the propionic acid found in the rumen.

6. The dietary history of the animal is shown to influence the rate at which glucose is fermented in the rumen, and the composition of the rumen microflora.

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BLOOD PRESSURE AND PULSE RATE IN THE FOETAL SHEEP

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(With Seven Text-figures)

BLOOD PRESSURE

Historical

Measurements of foetal blood pressure have been made from time to time by different authors, on different species, in different vessels and with widely differing results.

Ribemont (1879) gave the arterial pressure in the human foetus as 64 mm. Hg.

Cohnstein & Zuntz (1884) investigated the arterial pressure in sheep foetuses; their results varied in the umbilical artery from 84 to 39 mm. and in the umbilical vein from 34 to 16 mm. The difference varied from 51 to 14 mm. They naturally emphasized the high venous pressure, and thenceforward that legend has found much favour, probably because it lent mechanical support to the theory that the inferior caval blood was projected across the superior caval stream into the foramen orale.

More recently measurements of arterial pressure have been given by Schlossmann (1932) who obtained values of 50–60 mm. in the carotid of the goat, Clark (1932) 25–30 mm. in that of the cat (vagi cut), Haselhorst (1929) 46–110 mm. in that of man.

To the above must be added the elegant observations of Hamilton, Woodbury & Woods (1937), who give a figure of 50 mm. for the intraventricular systolic pressure in each ventricle of the dog's heart and similarly of 21 mm. in those of the rabbit.

The conception of an increase of arterial pressure with that of foetal age might be deduced from Cohnstein & Zuntz's (1884) results; they give the foetal weights but not the ages. The first author to state this conception was Seitz (1901).

Barcroft (1935) published the following results shown in Table 1 as measured by the mercurial manometer applied to the sheep's foetus:

Table 1

Foetal age, days	101	120	123	137	138	140
Carotid pressure, mm. Hg	34	46	50	72	68	76

In addition, the present authors have had the opportunity of seeing some beautiful observations obtained on chick embryos of various ages by A. F. W. Hughes.

Methods

Apparatus

The following method has been the standard method used in measuring the pressure of various foetal vessels.

For measurements of arterial pressure a hypodermic needle has been fitted with a piece of translucent bicycle valve tubing of about a metre in length, with a small glass funnel at the other end. Owing to war conditions it became impossible at one juncture to obtain translucent tubing, and therefore glass tubing of about 2 mm. bore was used, connected to the hypodermic needle by a short piece of valve tubing. The system was filled with 'Heparin Ringer' solution from the tip of the hypodermic needle to a distance up the tube necessary to form a column somewhat higher than represents the blood pressure measured in Ringer's solution. A 'bull-dog' forceps is put on the rubber tubing to prevent any passage of the fluid along it. Great care, of course, must be exercised lest any bubble of air enter the system. When the moment arrives to measure the blood pressure, the funnel is suspended at a height such that when the needle point is thrust into the vessel and the clip removed, 'Ringer' will run into the circulating blood and not the blood into the tubing. The meniscus in the tube shortly settles to a level which is constant but for the oscillations produced by the heart beat or otherwise. The pulse in the artery should be visible. The level of the meniscus, as compared with that of the foetal heart, is measured with an ordinary wooden scale held by hand. The measurement is rendered easier by the fact that the foetus is in a saline bath. The actual measurement made is that of the difference of level between the meniscus and the surface of the saline bath—a correction being applied for the depth to which the foetal heart is below the saline.

The points about this very simple method are:

(1) that it can be applied instantly;

(2) that it can be applied with the vessel in any position;

(3) it demands a minimal amount of manipulation of either the foetus or the vessel.

The measurement of blood pressure by the ordinary mercurial manometer on the other hand demands

(1) that the animal be still;
(2) that it be fixed in a suitable position;
(3) the insertion of a cannula in the vessel;
(4) the lapse of time necessary to secure the above manipulations.

The umbilical vessels, which are perhaps the most interesting from the present point of view, will not withstand these operative procedures. The reading must be taken without kinking or even constricting the vessel: the position of the foetus is unpredictable, and trauma of any sort to the vessel must be ruled out to the maximum possible extent. It follows then that the needle must be as sharp as possible (it is better to use a new needle for each measurement and that it should be as fine as is consistent with the flow of fluid through it not being unduly slow).

This will be called the 'needle method'.

Comparison with the mercurial manometer

The needle method and the mercurial manometer do not measure precisely the same thing. If the needle is 'small' relatively to the bore of the vessel into which it is inserted, it will measure the pressure in a stream of fluid, apart from a correction depending upon whether the orifice of the needle is directed towards or away from the stream. The mercurial manometer, on the other hand, is attached to a cannula in a closed vessel in which there is no stream, and measures the pressure not within the stream in that vessel, but on the wall of the one from which it is derived.

The question arises: what is the quantitative relation of the two systems of measurement. The answer can be obtained by estimating the pressure in one carotid artery by the mercurial manometer and in the other simultaneously with the needle. The results are given in Table 2.

Table 2. *Carotid pressure, mm.* Hg. *Sheep* 612. *Foetal age* 147 *days*

	a	*b*	*c*	*d*	*e*
Mercurial manometer, right carotid	77	79	80	82	76
Needle, left carotid, flow free	73	—	71	—	73
Needle, left carotid, flow stopped by clip distal to needle	—	79	—	83	—

When the carotid is clipped distally to the needle with consequent stoppage of the blood stream, the needle and the manometer are measuring the same thing and give substantially the same result, but when the current is flowing freely, the needle gives a recognizably lower reading than the manometer, the difference on the average of the above (*a*, *c* and *e*) being 5 mm. Hg.

The direction of the needle

It is desirable in taking a measurement to point the needle first towards the heart and then away from it. The difference in the result is illustrated in Table 3.

Table 3. *Blood pressure, mm.* Hg

Serial no. of sheep	Foetal age days	Umbilical artery Needle pointing towards		Umbilical vein Needle pointing towards	
		Heart	Placenta	Heart	Placenta
471	118	33·5	33·7	14·4	16·7
448	120	39·8	36·8	7·2	7·5

Clearly, the direction of the needle makes little difference to the measurement. The largest divergence above is 3 or 1·5 mm. from the mean, an amount well within the casual physiological errors of the experiment. Nevertheless, the dual reading is worth taking because it sometimes reveals a kink in the vessel.

Conditions

The data given in Table 4 were obtained under rigorous conditions of which the two principal were the exposure of the foetus to the least possible degree and for the least possible time. So far as degree of exposure is concerned, it must be remembered that the lower part of the body of the ewe is in a saline bath and in it the uterus is exposed. There are then two possible procedures:

(1) To open the uterus, rapidly deliver the foetus into the saline and insert the needle into the cord. This method has the advantage that the operator can see the cord from end to end and can assure himself that there is no pressure on either the foetus or on the cord.

(2) In the case of a singlet, to take advantage of the fact that the vessels from the cotyledons of both horns of the uterus contribute to the main umbilical vessels. The horn which does not contain the foetus is opened, and a medium-sized branch of the umbilical vessel is selected. The merit of this method is that the foetus is never even seen, let alone exposed: its drawback is lest some unsuspected pressure or tension on the main vessel should vitiate the result. Actually, we have used both methods without discovering any constant difference between the results which they furnish, but in any case speed is the essence of a satisfactory result.

A good check is the pulse. In the older foetuses the pulse may easily be felt through the uterine wall before the uterus is opened; if it is unaffected, there is probably no serious pressure on or kinking of the umbilical vessels.

Anaesthetics

We have used for the ewe spinal anaesthesia 3 c.c. of duracaine, supplemented if necessary by local anaesthesia. So far as the foetus is concerned there are no sensory nerves in the umbilical vessels, and if they only are the subject of experiment, we have used no anaesthetic for the foetus; where skin incisions have been undertaken we have used local anaesthetic for all foetuses over 133 days foetal age.

Results

The umbilical vessels

Table 4 shows the results obtained from the umbilical vessels by the needle technique.

Table 4. *Pressure in umbilical vessels measured by needle technique, mm.* Hg

Serial no. of sheep	Foetal age days	Pressure		
		Umbilical artery	Umbilical vein	Difference
428	43	6	—	—
452	48	6	—	—
432	58	20	5	15
562	59	19	7	12
478	59	32	13	19
567	73	22	7	15
554 (twins)	75	19	7	12
		30	11	19
484	76	22	15	7
426	84	28	7	21
463	89	—	10	—
570	90	32	7	25
584	93	34	—	—
554	106	37	—	—
481	111	32	6	26
445	115	31	6	25
471	118	34	15	19
448	120	36	14	22
663	129	38	8	30
664	131	38	11–12	21–26
642	136	—	11	—
643	136	—	9	—
427	141	48	9	39
654	142	52	—	—
656	143	51	14	37
657	144	58	18	40
611	146	51	—	—
612	147	46	—	—
563	149	44	—	—

The umbilical artery

The figures in Table 4 differ greatly from those in Table 1. The difference is trivial or non-existent up to ages around 100 days, but as gestation proceeds the disparity increases, so that at term it amounts to something of the order of 20–30 mm. As many more figures might be given confirming those in Table 1, an inquiry is necessary as to the discrepancy. We have already seen that the needle method gives lower readings than the manometer method by about 5 mm., which still leaves 15–25 mm. to be accounted for. The measurements in Table 4 are from the umbilical artery, whilst those in Table 1 are from the carotid. It is well known that in animals the size of a sheep's foetus at term (e.g. the cat or rabbit) there is no appreciable difference between the pressure in the carotid and that in the femoral artery as measured by the mercurial manometer The foetus, however, presents a rather special case: inserted in the systemic circulation between the heart and the femoral artery is the placental circulation providing a large outlet of blood into an area of low resistance. It might well be that such an addition to the circulation might cause an appreciable pressure gradient along the aorta and so cause the pressure in the femoral artery to be lower than that in the carotid. In fact, the pressure in the femoral artery of the sheep foetus is usually a few millimetres below that in the carotid, as shown on manometer tracings taken from the two vessels simultaneously. When a direct simultaneous comparison is made by the needle technique as between the pressure in the carotid artery and that in the umbilical artery, the former was the higher in four cases out of five, and the difference is trivial as compared with the 15–25 mm. which we have to explain. Moreover, for sheep 610, 611 and 612 in Table 5—all approaching term—the measurements in the umbilical artery are 67, 74 and 60 mm. respectively, whilst those of corresponding age 427, 611, 612, 563, in Table 4 are 44–51 mm. As two of the sheep, 611 and 612, are common to both Tables 4 and 5, and as the apparatus was the same in both sets of measurements, it is natural to refer the difference in the pressure to the conditions under which the measurements were taken.

Table 5. *Pressure in mm.* Hg, *needle technique*

Serial no.	Foetal age days	Carotid artery	Umbilical artery
581	85	31·6	30·3
585	95	46	43
610	143	71	67
611	146	70	74
612	147	73	60

Those in Table 4 were taken either with the foetus in the uterus or immediately after its delivery; those in Table 5 were taken necessarily after a lapse of time long enough to dissect out and cannulize the carotid and, what is perhaps as important as the time lag, after a subjection of the foetus to all the sensory influences consequent on such an experiment. Indeed, in foetus 611 the mean pressure continued to rise gradually for a considerable time after the first record of carotid pressure was taken. Fig. 2 (1)–(4) shows the alterations in blood pressure as time elapsed. The pressure, as shown by the needle before the dissection commenced, was, as stated in Table 4, 51 mm. Hg.

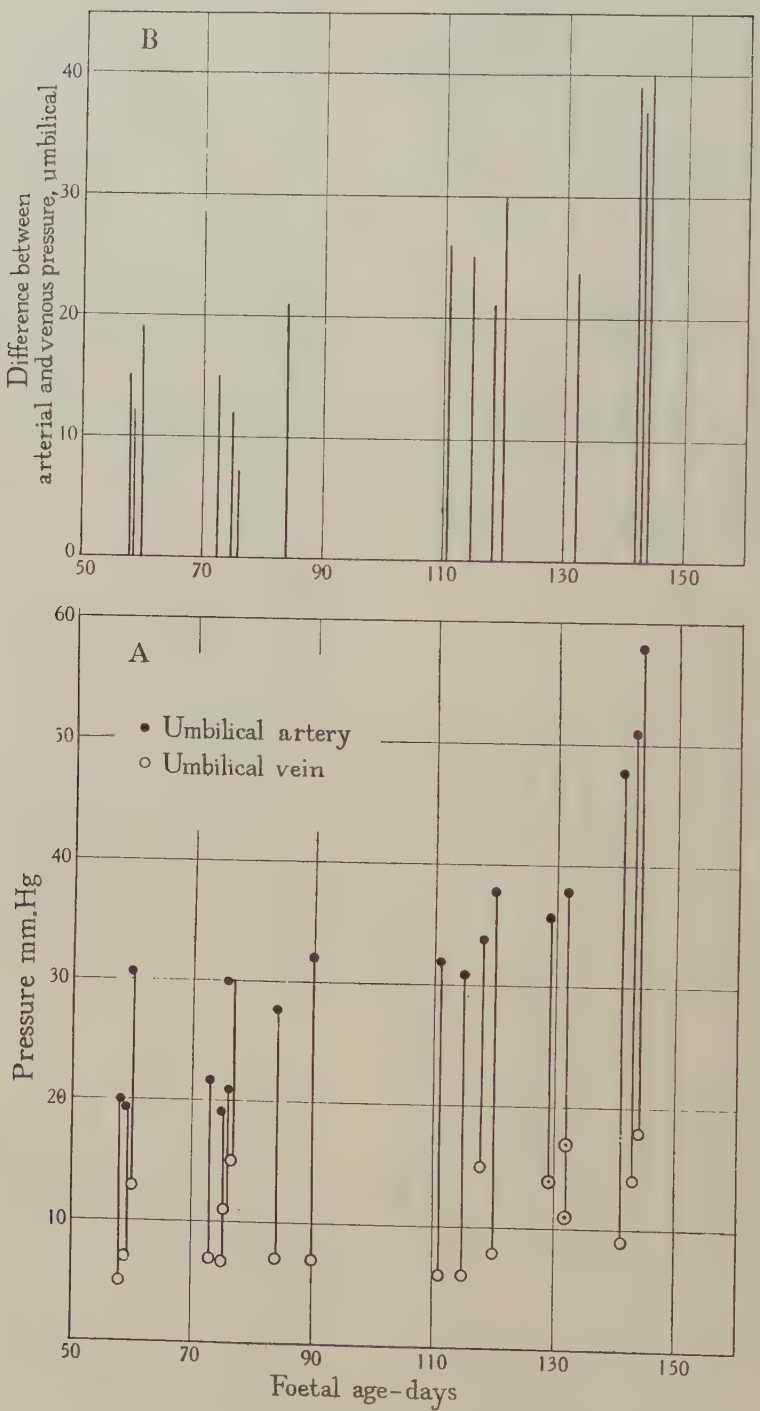

Fig. 1. A. Blood pressure in umbilical vessels considered in respect of foetal age. B. Difference of pressure as between umbilical artery and vein considered in respect of foetal age.

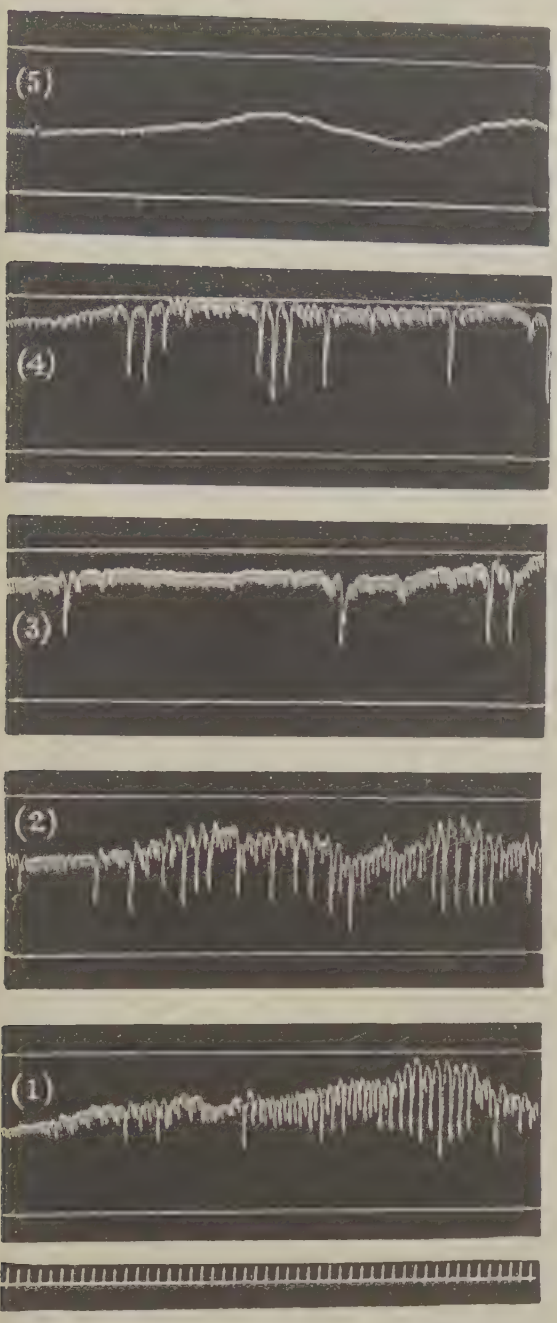

Fig. 2. Carotid blood pressure. Records taken at intervals of about 3 min. The lower horizontal line in each tracing corresponds to 50 mm., the upper line to 90 mm. Between tracings (4) and (5) both vagi were cut. Time = seconds.

So far, therefore, as experimental procedure allows, we believe that the figures given in Table 4 present the most exact picture at present available (see also Fig. 1) of the pressure in the umbilical vessels, as these vessels exist in the uterus: in short, that during intra-uterine life the pressure in the umbilical artery gradually rises from 6 mm. Hg on the 43rd day to 50–60 at term.

The carotid artery

The surest estimate of the carotid pressure of the sheep's foetus *in utero* appears to be an indrect one, not to try to measure it directly as several workers have done, but to take it as being a few millimetres higher (see Table 5) than the pressure in the umbilical artery.

The umbilical vein

According to Table 4 there is, at any age, a considerable variation in the range of observed venous pressures (Fig. 1).

Table 6. *Range of pressures in umbilical vein at different foetal ages*

Foetal age, days	50–74	75–99	100–124	125–149
Venous pressure, mm. Hg	5–13	7–15	6– 15	8– 18

Whilst the range of pressure is considerable, the highest in each age group being more than twice the lowest, the mean venous pressure varies little over the period from 58 days onwards; it seems, however, to rise in the last week. At this age the actual fluctuations from moment to moment are quite considerable; clearly, they may be due to events happening in the uterus, in the foetus, or both. Any strong muscular movement in the foetus sends up the arterial pressure; the effect on the venous pressure has not been registered. In one foetus (sheep 644, 130 days) four pressure waves were observed in 1½ min., the maximal pressures being respectively 14, 16, 17, 17 mm. Hg, and the minimal pressures 13, 12, 12, 14. The cause of these waves awaits further investigation.

Discussion

It seems possible now to reduce the results obtained by previous workers to some degree of order.

Evidently the arterial blood pressure is a function of the degree of development of the foetus. Those forms which are born in an immature condition, such as the rabbit (b.p. 21 mm.) and the cat (b.p. 25–30 mm.), have blood pressures very similar to what the sheep foetus would have at a corresponding degree of development.

When, on the other hand, we consider the hitherto published figures for the sheep and the goat at the end of gestation, clearly these record higher pressures than those which obtain during intra-uterine life: and that, because the foetuses in question were in conditions of exposure intermediate between those of general external stimulation and those of relative sensory vacuum which obtains during intra-uterine life.

The figures obtained for pressure in the umbilical veins are much lower than those obtained by Cohnstein & Zuntz (1884). We attribute their high values to constriction of the umbilical veins, especially on the umbilical side of the cannulae which they inserted into these vessels, with consequent rise of pressure in the placental vessels.

The work of Barclay, Franklin & Prichard (1944) illuminates the functions of the great veins in the vicinity of the liver in the sheep. The umbilical vein and the portal vein meet to form an arcade from which several veins branch: of these one is the ductus venosus, others go into the liver, and the blood from them reaches the branches of the hepatic vein. It is therefore natural to suppose that the pressures in the umbilical and portal veins are not very unequal, and are influenced by the bore at any time of the ductus venosus which is controlled by the vagus. Unfortunately, we have no measurements of the portal vein in the foetus.

PULSE RATE

Historical

A number of studies have been made of the foetal pulse. The literature will be found in Windle's book (1940), or in Bogue's (1933) paper on the pulse rate in the chick. The general conclusion in the literature is that as the period of gestation proceeds, the pulse rate quickens, though according to Bogue's figures for the chick, obtained by the electrocardiogram, the pulse rate in the last few days before hatching is pretty constant. Cohnstein & Zuntz (1884) say, 'The frequency of the pulse appears to be greater in the younger foetuses (1200–1500 g.) than in the full-time foetuses of 3600 g. In the former the lowest value was 114 after severe bleeding and the highest 210; in the mature foetus the values ranged from 77 to 125.' Judged on the scale of Welsh sheep such as I have used, 1200–1500 g. would correspond to about 120 days foetal age and 3600 would be approaching term (147 days). According to these authors, therefore (apart from the bleeding), the pulse of the sheep, unlike those of other recorded forms, fall in the last 3 weeks of gestation to about half its former value. The observations of Cohnstein & Zuntz (1884) were made by means of a mercurial manometer attached to one of the umbilical arteries. It is not difficult, by using similar methods—e.g. the registration of blood pressure by a mercurial manometer connected with the carotid of a sheep foetus—to obtain results similar to those of Cohnstein & Zuntz (1884). Such a result, however, demands some explanation.

In what follows we may make a preliminary survey of the relation of the pulse rate to the foetal age under four sets of conditions:

I. *In utero.*
II. Immediately on delivery of the foetus.
III. After severance of both foetal vagi.
IV. When driven at its maximum rate with adrenalin.

I. The pulse of the foetus *in utero*

Methods

The usual mercurial manometer technique as a method of measuring the foetal pulse was very soon discarded; the pulse rate given by it obviously had little relation to the actual pulse rate *in utero*. This is perhaps not surprising: the manometer as usually used involves the insertion of a cannula into some artery, which again involves a certain amount of exposure, of dissection and a considerable lapse of time.

Our approach was an endeavour to count the foetal pulse without opening and, if possible, without displacing the uterus.

Three obvious ways of counting the intra-uterine foetal pulse exist:

(1) By auscultation through the abdominal wall as is done in man.
(2) By palpation of the uterus.
(3) By the use of an electrocardiogram.

Of these the first is very difficult in the sheep, largely because of its woolly coat and perhaps on account of the thickness of its skin, both foetal and maternal. At all events, it was often impossible to hear the foetal pulse by application of the stethoscope to the abdominal wall of the ewe.

The second method proved satisfactory in every respect except that it necessitated the opening of the abdomen and the sacrifice of the ewe.

The third method, which in theory has much to commend it, we did not try, in view of the ease with which the second could be applied.

The ewe is given a spinal anaesthetic and is placed with the lower part of the body in a bath of saline at 39–40° C. An incision is made into the abdominal wall in the middle line, the hand is passed in and the pulse easily felt through the uterine wall. Care, of course, is taken not to confuse the foetal pulse with that of the mother and, what is sometimes more difficult, not to confuse it with the respiration of the mother. This method can be used after about 90 days foetal age, and by means of it the figures given in column 3, Table 7, were obtained. In spite of considerable variations from animal to animal, the general tendency during the last 50 days of gestation undoubtedly is for the intra-uterine pulse rate to get slower. There is, however, no such great difference between 120 days and term as Cohnstein & Zuntz (1884) observed, nor is the pulse rate at 120 days anything like 210 beats to the minute.

II. The pulse immediately on delivery

When the uterus is opened and the foetus is withdrawn into the bath, whatever care be taken not to interfere with the circulation, there is usually a tem-

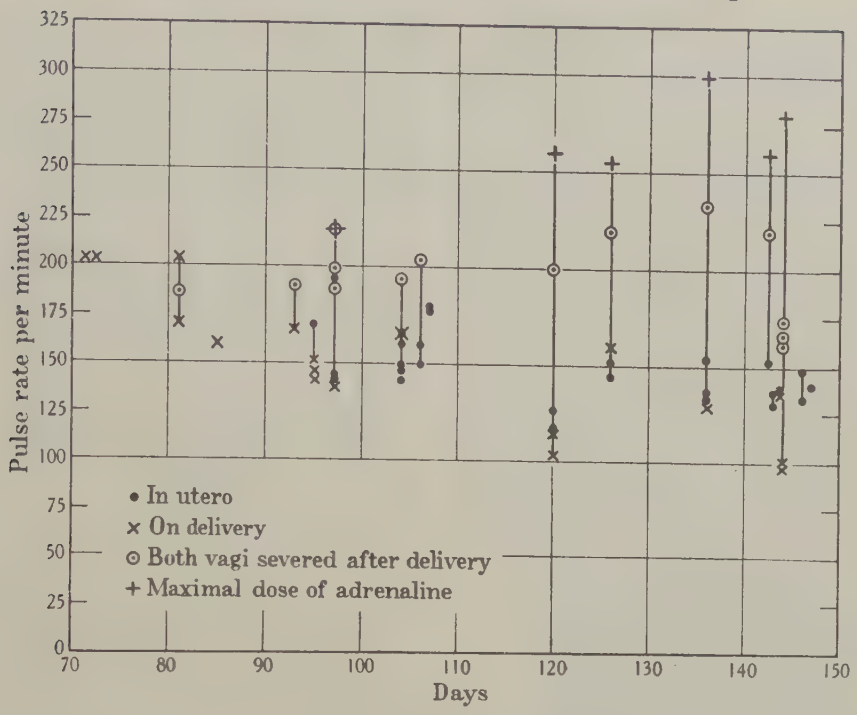

Fig. 3. Pulse rate considered in respect of foetal age.

Table 7. *Recognizable levels of foetal pulse in sheep (beats per minute)*

1	2	3	4	5	6
Serial no. of sheep	Foetal age days	*In utero*	Just after delivery	Just after section of vagi	Strong dose adrenalin, maximal rate maintained for 15 sec.
63	32	—	80	—	—
126	34	—	108	—	—
116	42	—	112	—	—
121	43	—	126	—	—
122	45	—	124	—	—
100	48	—	192	—	—
591	77	—	186	—	—
596	80	—	152	—	—
577	81	—	204–168	166	—
581	85	—	164	172	—
590	90	170	—	—	—
604	97	143	138	200	220
593	104	152	166	194	—
584	108	134	138	227	248
599	120	129	104	200	260
592	126	155	162	220	256
598	136	—	112	—	—
610	143	134	130	—	—
605	144	107	102	165	280
611	146	141–134	108	—	—

porary upset in the pulse which may last for half a minute or so. This commonly takes the form of a considerable slowing, but after about half a minute the pulse generally (though not in every case) settles down to within ten or fifteen beats of the intra-uterine value (Table 7, cp. columns 3 and 4). It is, of course, easy then to count the pulse either by inspection of the cord or palpation of the chest.

Before 90 days we are dependent for our observations on pulse rates which have been obtained in this way. They will be found in column 4, Table 7, though most of these observations were made with only such interference as was wrought by delivering the sac from the uterus, the foetus being still in the sac with its circulation intact. Judged by the figures in column 4, the pulse rate in the sheep reaches a maximum value of about 200 from which, after about the 12th week of foetal life, it recedes until at term the pulse may not be much over 100 beats to the minute.

Here we are approaching the phenomenon which Cohnstein & Zuntz observed, except that they described the pulse rate as maintained at 210 at 120 days, by which time, according to our observations, it is already very much slowed.

III. After severance of the vagi

That the slowing of the pulse during the last third of gestation is due to the establishment of vagus tone is shown by the effect of cutting the vagi. The figures in column 5 are those obtained by cutting both vagi

as soon as possible after taking the observations recorded in column 4. Up to 85 days there is no evidence of considerable slowing of the heart on section of the vagi, but at 97 days the amount of vagus inhibition *in utero* appears to be considerable, and on the whole the degree of inhibition increases until the end of the gestation period. As might be expected there is a certain irregularity in the observations made in the uterus, the hearts of some foetuses being inhibited to a greater degree than those of others. There are certain anomalies, also, such as foetus 605 in which the figures in columns 3, 4 and 5 are all very low. The inhibition indicated in columns 3 and 4, judged as a percentage of the rate with the vagi cut (column 5; see also Fig. 3), is not abnormal. Such irregularities have not been pursued. They are the bane, so far as the author's experience goes, of all research on pulse rates whether in foetuses or human beings: some persons seem to have hearts that are basally slower than others. But such cases do raise the question whether, when the vagi are cut, the heart is still beating against a background of hormonal control, notably that exerted by adrenalin.

IV. After large doses of adrenalin

Some experiments were carried out, therefore, to test (1) whether the foetal heart with the vagi cut was capable of a further acceleration as the result of the injection of adrenalin: (2) if so, what relation this maximal heart rate had, if any, to the rate registered after section of the vagi.

Table 8. *Pulse rate after injection of* ½ *c.c.* 1/1000 *adrenalin*

Serial no. of sheep	Foetal age days	Pulse rate in successive ¼ min. intervals											
		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
604	97	50	55	54	52	39	54	35	34	40	41	40	42
592	126	64	32	65	54	48	48	38	40	36	37	—	—
605	144	44	65	67	68	70	70	70	66	69	—	—	—

The first question was answered at once: injection of a heavy dose of adrenalin does produce an acceleration of the pulse after the vagi are cut. The second question was not answered completely; the most that can be said (see Fig. 3) is (*a*) that there is a general tendency for the maximal rate to rise as term approaches; (*b*) that the low pulses which prevail in foetus 605, both when the vagus is intact and when it is cut, find no counterpart in the adrenalin value.

The second conclusion, however, is subject to an over-ruling factor which might be held to make the whole comparison unreal, namely, that whilst the pulse rate both before and after vagotomy is sustained, that which results from a maximal dose of adrenalin is not. The figures in column 6 have been sustained for at least 15 sec.

After the first minute the pulse in the younger foetuses tend to become very irregular (604). Three samples are given—592 is interesting, the figures appearing more irregular than those of the other two, but possibly the irregularity is less than might appear because the rate in the second period is just half that in the first and third. It could be argued that the auricular beat was probably just over 64 and that the ventricle was beating at a 1:2 rhythm.

Establishment of vagus control of the pulse

The experiments described above show that the foetal pulse is under vagus control after 90–100 days even when the foetus is in the uterus. They do not, however, indicate whether this control is central or reflex.

Bauer (1938, 1939) showed that in the rabbit asphyxia caused vagal slowing of the heart on the 11th day after birth, whilst it was not until the 30th day that the depressor effect is elicited reflexly by asphyxia.

It seemed desirable to ascertain for the sheep the earliest date at which stimulation of the central end of one vagus (the left) could evoke cardiac inhibition, the right vagus being intact. Clearly, if this date was after the onset of intra-uterine vagus slowing, the latter phenomenon must at its initiation be regarded as central. If, on the other hand, the reflex machinery was demonstrable at an earlier date than that at which the normal bradycardia developed, the question still remained open; the bradycardia might be either central or reflex. In this event it proved possible to inhibit the heart by stimulation of the central end of the vagus on the 77th day. Indeed, it is an open question whether the central machinery is not in being as early as the terminal vagal motor mechanism in the heart.

Bauer (1937) showed that on the 88th day stimulation of the peripheral end of the right vagus produced

Rabbit 577, 81 *days*

84 beats in 30 sec.		No stimulation, left vagus (*L.V.*) cut
79	,,	Peripheral end of *L.V.* stimulated
77	,,	No stimulation
82	,,	Peripheral end of *L.V.* stimulated, right vagus (*R.V.*) cut
83	,,	No stimulation
46	,,	Peripheral end of *R.V.* stimulated
84	,,	No stimulation
50	,,	Peripheral end of *R.V.* stimulated
68	,,	Peripheral end of *L.V.* stimulated
83	,,	No stimulation
78	,,	Peripheral end of *L.V.* stimulated

a definite slowing of the heart; the left vagus in his experiment gave no slowing. This I have confirmed but at 81 days.

In the above experiment on the two occasions on which the right vagus was stimulated the pulse rate was reduced to between two-thirds and a half of its former rate, whilst only in one out of four cases in which the left vagus was stimulated was there any reduction in pulse rate and that was so slight as to be of uncertain significance.

This observation has an important bearing on the experiments which follow. To demonstrate slowing of the heart at this age (should it occur) as the result of central stimulation of the vagus, the right vagus must be left intact and the central end of the left vagus stimulated.

In an experiment at 85 days (sheep 581) I also failed to obtain cardiac inhibition on stimulation of the peripheral end of the left vagus.

By the 101st day, if not before, stimulation of the peripheral end of the left vagus will produce slowing of the heart rate.

Sheep 523, 101 days.

No stimulation, 15 beats in 5 sec.

Stimulation of peripheral end of left vagus 12, 12, 12, 12, 12, 12, 11, 11 beats in successive 5 sec. intervals.

Cease stimulation 13, 15 in next two periods.

In the records of this laboratory there is a statement in Barron's handwriting stating that he had produced a vagal slowing of the sheep's foetal heart at 77 days by peripheral stimulation. This I have confirmed and I have extended the observation to the point of having also obtained slowing of the pulse rate at this age by stimulation of the central end of the left vagus, the right vagus being, of course, intact.

The following is the protocol of the experiment:

Sheep 591—77 days—spinal anaesthesia (3 c.c. duracaine).

Experiment conducted with sheep in saline bath.

Foetal pulse	Remarks
93 in 30 sec.	On delivery
	Vagi dissected out
	Left vagus (*L.V.*) cut
39 in 15 sec.	No stimulation, pulse regular
39 in 15 sec.	No stimulation, pulse regular
64 in 30 sec.	Stimulation of central end of *L.V.*
	Beats in successive 5 sec. intervals 12, 14, 8, 10, 9, 11 = 64
59 in 30 sec.	Stimulation of central end of *L.V.*
	Beats in successive 5 sec. intervals 15, 11, 10, 8, 7, 8 = 59
64 in 30 sec.	Stimulation of central end of *L.V.*
	Beats in successive 5 sec. intervals 14, 12, 10, 10, 8, 10 = 64
45 in 15 sec. (16, 14, 15)	No stimulation
	Right vagus (*R.V.*) cut
38 in 15 sec. (14, 12, 12)	No stimulation
26 in 15 sec. (10, 8, 8)	Peripheral end of *R.V.* stimulated
24 in 15 sec. (9, 8, 7)	Peripheral end of *R.V.* stimulated
42 in 15 sec. (15, 14, 13)	No stimulation

The above showed evidence both of reflex slowing of the heart when the central end of the left vagus was stimulated, the right vagus being intact, and of direct slowing when the distal end of the right vagus was stimulated subsequently. The reflex slowing in each case developed gradually, whilst the inhibition due to direct stimulation appeared at once.

In an experiment, however, at 81 days the reflex slowing was only obtained on one out of four attempts, whilst the slowing by peripheral stimulation was obtained on each occasion on which it was tried.

At 83 days a tracing was obtained (sheep 586) which, when magnified, showed the pulse quite plainly and indicated a slowing of the heart on stimulation of the central end of the left vagus from a steady rate of 12–13 beats per 5 sec. to 9½ beats.

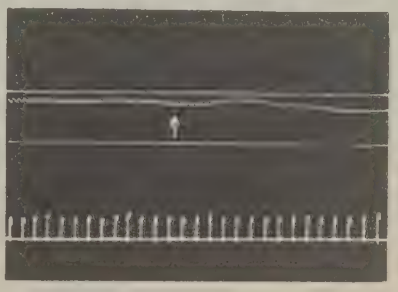

Fig. 4. Foetus 568, 83 days, tracing of arterial pressure. Tracing magnified ×2 (approx.). Arrow marks commencement of stimulation of central end of left vagus. Upper horizontal line = 50 mm. Hg; lower line = 40 mm. Time = seconds.

Cardiac inhibition has been obtained, therefore, on the 77th day either by direct stimulation of the right, but not the left, vagus and by central stimulation of the left vagus.

Because the machinery for reflex cardiac inhibition is in being, there can be no assumption that use is being made of it. Indeed, Bauer (1939), in the rabbit, found that the heart could be slowed by electrical stimulation of the carotid sinus, and depressor nerves produced slowing of the heart on the 13th and 11th days of post-natal life, whilst they were not used physiologically till the 30th day.

Slowing of the heart associated with rise of blood pressure has been observed as early as 111 days (sheep 563) (Fig. 5). The heart had been beating regularly for some time, as at the commencement of the tracing the systolic pressure rose from 52 to 62 mm. Hg.

Slowing of the heart has been demonstrated as the result of an experimentally produced rise of blood pressure at about 120 days, whether the rise of

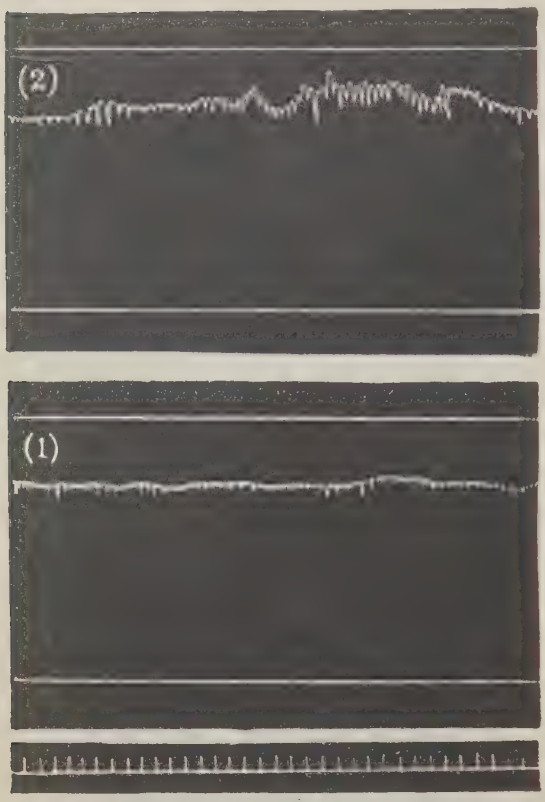

Fig. 5. Arterial pressure, foetus 111 days, tracing 2 followed directly on 1. Lower horizontal line = 0; upper line = 70 mm. Time = seconds.

pressure may be sudden, due to clamping the umbilical cord, or gradual, due to the injection of adrenalin.

(*a*) By clamping the cord (sheep 521, 121 days)

(1) Beats in 5 sec.	19	19	19*	16½	16	15	15	15
intervals								
A.B.P. mm.	20	20	20	20–26	26	26	26	26
(2) Beats in 5 sec.	16	16	16	16*	14	14	14½	
intervals								
A.B.P. mm.	40	40	40	40–45	45	45	45	

* Cord clamped between these points.

The fact that the slowing is obvious within 5 sec. is the evidence of its being a reflex effect and not one due to asphyxia.

The effect of clamping the cord, as given in sheep 521, was verified on 526.

(*b*) By injection of adrenalin (sheep 526, 118 days)

Before the injection the heart was going at a regular rate of 22–23 beats in 5 sec.; the injection consisted of 1 c.c. of 1/50,000 adrenalin into the umbilical cord. In the first two subsequent 5 sec. intervals the B.P. rose from 35 to 44 mm. without any alteration in the pulse rate. The pulse abruptly slowed to 18, 17, 16, 16, 16, with an obvious increase in the stroke volume. During those 25 sec. the B.P. rose another 6 mm. Hg. The tracing might be interpreted as indicating that 44 mm. was the limiting pressure at which the cardiac depressor (and/or carotid sinus) commenced to operate.

The above observation was repeated with substantially the same result.

That the slowing of the pulse caused by the injection of adrenalin is a vagal phenomena is shown in Fig. 6 in which three injections of adrenalin were made (sheep 540, dose 1 c.c.). In the first and third records there is an obvious slowing of the pulse of 1/50,000; when the second was made the vagi were blocked by the insertion underneath them of ice-

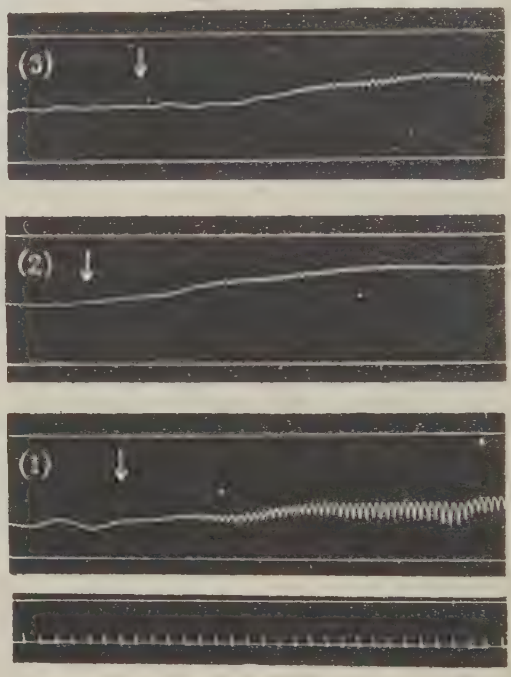

Fig. 6. Arterial pressure sheep 540, 143 days, effect of intravenous (jugular) injection of 1 c.c. of 1/50,000 adrenalin (arrow) (1) vagi not blocked, (2) vagi blocked by ice-cooled plates, (3) vagi not blocked. Lower horizontal line = 50 mm.; upper line = 100 mm. Time = seconds.

cooled copper plates, the pulse was scarcely, if at all, affected by the injection.

Hitherto there has been no satisfactory information concerning the degree to which the cardiac depressor fibres and the carotid sinus respectively are involved in the reflex slowing of the heart. In the experiments quoted at about 120 days, ligature of both carotids did not abolish the vagus inhibition, but unfortunately ligature of both carotids is not a sufficiently rigorous experimental procedure. On several occasions we have abolished the vagus inhibition by removal of or destruction of the carotid sinus on both sides. This procedure is, however, open to objection; it may be argued that by the time so drastic an operation has been made, the central

nervous system of the foetus will have lost its irritability. Only in one experiment have we satisfactorily demonstrated the action of the carotid sinus in the foetus.

Sheep 534, 154 days:

(1) The carotid and vagi were dissected out in the neck and threads put loosely round them.

(2) The carotid bifurcations were dissected out and the internal and external carotids were ligated about 7 mm. from the bifurcation, no visible nervous tissue being included in the ligatures.

(3) Cannula for arterial pressure in femoral artery and tracing commenced: systolic pressure 70 mm., pulse 11½ in 5 sec., left carotid clamped, pulse rose to 13: right carotid clamped pulse rose to 15: clamps removed pulse fell to 12½.

(4) The cord was clamped so as to raise the blood pressure, the rise was 45 mm., the pulse dropped to 8, the right carotid was clamped and the pulse rose to 10: the left carotid was clamped and the pulse rose further to 12 in 5 sec.

(5) Both vagi were cut: the pulse rose to 19.

Counted by palpation the operator would be satisfied that the pulse has slowed and would probably be happy to give a precise figure for the pulse rate. From the tracing, however, it is not quite clear, in terms of auricular and ventricular beats, what has happened. In order to be clear an analysis of the phenomenon with the string galvanometer is required and will be undertaken. The established facts about the response are (1) it is typical, (2) it is a response to rising pressure, (3) it depends upon the integrity of the vagi, (4) it results in the return of the pressure to its former level.

The tracings in Fig. 2 were taken at intervals of about 3 min. The whole figure therefore covers about a quarter of an hour and tracings 1–4, in which the vagi are intact, about 12 min. In this time the general level has risen to about 90 mm. Hg. The pressure started at 51, when the uterus was opened: it was 61 at the commencement of tracing 1. Simultaneously with the general rise of pressure, which in tracing 4 was nearly 90 mm. Hg, there was a falling off in the vagus response.

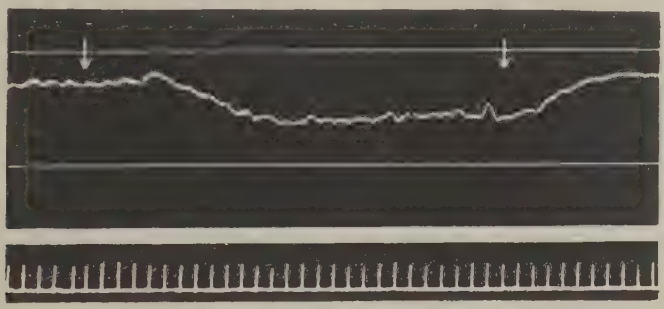

Fig. 7. Arterial pressure, sheep 606, 142 days. Arrows denote commencement and end of stimulation of central end of left vagus, right vagus cut, horizontal lines 60 and 80 mm. Hg respectively. Time = seconds. × 2.

The above shows evidence of carotid sinus control of the pulse mild in effect at the lower pressure (para. 3 above) and powerful at the higher pressure (para. 4): also of cardiac depressor action, inasmuch as when the carotid sinus was cut out, the pulse remained much below 19 (para. 5).

Fig. 2, tracing 1, gives a good idea of the effect of rise of blood pressure in a foetus shortly before term. The response is a powerful vagus reaction. The pulse at the commencement was regular, the rate being 15½ in 5 sec.; it was under some degree of vagus inhibition as the rate rose to 20 in 5 sec. when both vagi were cut (tracing 5). After a slight rise in pressure, accompanied by a slight slowing, the pulse underwent marked alteration. For the moment I will merely call this the 'vagus response' because it is never seen on any tracing after the vagi have been cut. It is followed by and presumably causes a return of the pressure to somewhere near its former level. I have used the phrase 'vagus response' rather than 'example of Marey's Law' because the counting of the pulse in this phenomenon presents difficulties.

THE ESTABLISHMENT OF VASO-MOTOR TONE

Not much can be said as yet about the possibility of vaso-motor tone existing, or even potentially existing, in the foetus. In Fig. 4 a fall of arterial pressure appears on stimulation of the central end of the vagus. Without very substantial confirmation this could not be accepted as evidence of a true depressor inhibition of vaso-motor tone. There is quite definite evidence, however, of that phenomenon at a late stage of gestation as shown in Fig. 7. The experiment ran as follows:

Sheep 606, 142 days.

The central end of the left vagus was stimulated on three successive occasions; on the first two the right vagus was intact: in each case there was a fall of arterial pressure from 78 to 62 mm. and a slowing of the heart from 22 in 5 sec. to 14½ in the first and from 21 in 5 sec. to 15 in 5 sec. in the second, whilst the blood pressure dropped from 78 to 68 mm. The right vagus was then cut, and on stimulation of

the central end of the left vagus the heart did not slow, but the blood pressure fell (Fig. 7) from 75 to 68 mm.

SUMMARY AND CONCLUSIONS

1. A method (the needle method) is described for the measurement of the pressure in the stream going through a vessel.

2. In the foetal sheep the needle method applied to the umbilical artery gives substantially the same results as the mercurial manometer applied to the carotid, until about half-way through the gestation period.

3. As gestation proceeds the needle method applied at the first moment at which it can be applied to the umbilical artery (or a branch) gives readings substantially lower, and increasingly lower as gestation proceeds, than does the mercurial manometer read at the first moment at which it can be read.

4. The discrepancy is due to the sum of a number of causes which are discussed, but of these the most important is an actual rise of pressure between the time of delivery and the completion of the dissections contingent on the use of the mercurial manometer.

5. The cause of this is not at present demonstrated, but either or both of two factors may be concerned: (*a*) a dulling of the central nervous system which weakens the depressor reflex; (*b*) the establishment of a greater degree of vasomotor tone consequent on the bombardment of the central nervous system with sensory stimuli.

6. The pulse rates *in utero* and just after delivery of the foetus into a saline bath at 39–40° C. (the umbilical circulation being unimpaired) are not significantly different.

7. The pulse rate quickens up to the 70th–80th day, after which it becomes slower as gestation proceeds.

8. If both vagi be severed, the pulse rate tends to quicken throughout gestation. The pulse, therefore, comes increasingly under vagus inhibition from the 80th–90th day onwards.

9. Even after the vagi have been cut after the 120th day (it has not been tried before) adrenalin in sufficient quantity will cause a further quickening of the pulse.

10. The earliest date at which stimulation of the peripheral end of the right vagus was observed to slow the heart was the 77th day. On the 85th day peripheral stimulation of the left vagus also failed, but succeeded on the 101st day.

11. Central stimulation of the left vagus, with the right vagus intact, produced slowing on the 77th day.

12. Slowing of the heart synchronous with rise of arterial pressure has been observed on the 111th day.

13. Slowing of the heart which bears evidence of being reflex has been obtained by raising the blood pressure (clamping the cord) on the 121st day and by injection of adrenalin on the 118th day.

14. Approaching term both the carotid sinus and cardiac depressor mechanisms are functional.

15. Lowering of the blood pressure as the result of stimulation of the central end of the vagus and with both vagi severed can be demonstrated late in gestation.

We take this opportunity of thanking Dr Forsham and Dr D. J. Bauer, who were in one way or other associated with us at the commencement of this research: indeed, it grew out of experiments in which they participated. Dr A. T. Phillipson and Mr A. Carlyle, M.R.C.V.S., also gave us much help. The earlier experiments in the series and those which led up to them were financed by the Rockefeller Foundation, to whom our thanks are also due.

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THE MECHANISM OF ABSORPTION FROM THE RUMEN AS EXEMPLIFIED BY THE BEHAVIOUR OF ACETIC, PROPIONIC AND BUTYRIC ACIDS

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(With One Text-figure)

INTRODUCTION

Absorption of the lower fatty acids occurs directly from the rumen of sheep, in which organ they are produced as a result of bacterial fermentation. Previous work on this subject, in which the *p*H of the rumen was not controlled (Barcroft, McAnally & Phillipson, 1944), showed that:

(*a*) The quantity of acid absorbed is substantial.

(*b*) The rate of absorption from the normally filled rumen bears no simple relation to the concentration of volatile acid in the ingesta.

(*c*) When equimolar solutions of the sodium salts of acetic, propionic and butyric acids are placed in the rumen, they are absorbed at different rates. The smaller molecules are absorbed more rapidly than the larger ones.

The present research deals with the relative rates at which these three substances leave the rumen by passage through its stratified squamous epithelial lining. Normally the *p*H of the ingesta of the rumen remains comparatively stable in spite of the fermentations that proceed within it, and the reaction of the ingesta rarely exceeds the limits of *p*H 5·8–7·5, the average value being in the vicinity of *p*H 6·4 (Phillipson, 1942). This is an indication that the quantity of free acid present is never excessive. The proportions of the individual acids also remain fairly stable and are in the regions of acetic 65 %, propionic 20 % and butyric 15 % (Elsden, 1945). These physiological facts have been taken as the basis for the design of the present experiments. At *p*H 6·4 both fatty acid anion (A^-) and free acid (HA) are present in significant quantities, so that either or both of these may be passing through the rumen wall. By keeping the rumen contents at *p*H 7·5 the proportion of free acid is reduced to negligible proportions, and the permeability of the rumen wall to fatty acid anion may be studied. Then by changing the rumen *p*H to say 5·8, where a substantial amount of free acid is present, the permeability to both anion and free acid can be studied, and the loss of material as free acid readily calculated with knowledge of the permeability to anions provided by the first half of the experiment. The permeabilities to free acid and to anion can then be examined by the methods given by Danielli (1941, 1943) to elucidate the nature of the process of passage through the rumen epithelium.

METHODS

(1) *Operative procedures*

Adult ewes were used which had been provided with permanent rumen fistulae for at least 4 weeks. Food was withheld for 24 hr. before the experiment to facilitate emptying of the rumen, which was done by repeated washings with water at body temperature, either immediately before the experiment, or on the afternoon preceding the experiment. In the latter case the rumen was filled with a solution of artificial saliva to prevent it from becoming dry.*

The sheep were anaesthetized by the intravenous injection of nembutal, and the abdomen was opened to the right of the midline in the epigastric region. A ligature was passed round the abomasum, excluding the epiploic vessels, immediately below the omasum. The oesophagus was ligated in the cervical region, and a purse string suture was tied round the neck of the cannula in the rumen if there was any danger of leakage occurring at this point. These measures were taken to prevent any escape from the rumen and reticulum except by means of the cannula. In most animals a tracheal cannula was inserted.

(2) *Experimental procedure*

A solution of the sodium salts of acetic, propionic and butyric acids was used in which the acids were present either in the proportions in which they occur in the rumen or else in approximately equimolar

* The solution was devised by Dr E. I. McDougall from the results of analyses of the saliva of sheep. Its composition was as follows: $NaHCO_3$, 9·24 g. per litre; KCl, 0·45 g. per litre; $MgCl_2$, 0·047 g. per litre; $CaCl_2$, 0·055 g. per litre; $Na_2HPO_4(12H_2O)$, 7·125 g. per litre.

concentrations. The latter procedure was adopted to facilitate analysis. In all cases the concentration of the solution at the start of the experiment was 0·6 *M*.

The solution was introduced into the empty rumen at a *p*H of 7·4; at the end of 2 hr. the *p*H was altered to 5·8 by the addition of normal acetic acid. The addition of acid to the fluid in the rumen served a double purpose in that, besides reducing the *p*H, it allowed the volume of the rumen contents to be calculated by determining the concentration of acid in the rumen immediately before the alteration in *p*H and 5 min. afterwards. Five minutes were allowed for the fluid introduced to mix with the fluid already present, a process that was assisted by massage of the abdomen. Assuming that the quantity of acid absorbed during the 5 min. interval was negligible, the total volume of fluid in the rumen was calculated from the dilution of the known quantity of acid added. In two experiments the procedure was reversed, and the solution introduced into the rumen at the beginning of the experiment had a *p*H of 5·8 which was changed to *p*H 7·4 after 2 hr. by the addition of normal sodium hydroxide.

The *p*H of the solution, during the acid and neutral phase, was controlled by frequent determinations made with a glass electrode. Acid or alkali was added in normal solution as required to maintain the *p*H at a constant level. After every addition the fluid in the rumen was mixed by massaging the abdomen.

(3) *Analytical methods*

Total volatile acid was determined by steam distillation after the addition of a suitable quantity of the acidified magnesium sulphate solution described by McAnally (1944). The distillates were titrated with standard 0·02 or 0·01 potassium hydroxide under CO_2-free conditions.

The proportions of the individual acids in the distillates so obtained were determined by the partition chromatographic method introduced by Elsden (1945).

Sodium was estimated by the pyro-antimonate method of Kramer & Gittleman described by Peters & van Slyke (1932).

RESULTS

Table 1 shows the protocols of a typical experiment. The results obtained in three experiments are shown in Tables 2–4. In the experiment recorded in Tables 1 and 2 the initial volume was 2600 ml. and the final volume was 3800 ml. The samples taken from the rumen amounted only to 2 ml. on each occasion and have been neglected in subsequent calculations. The initial dilution of the dose by the residual fluid left in the rumen before it was introduced can be calculated by the change in concentration of the total acid; thus in this experiment, when 2600 ml. of solution *X* containing 32·8 ml. normal acids % were placed in the rumen, the concentration of acid fell to 31·2 ml. normal acids %. The true volume of the rumen contents at the beginning of the experiment therefore was

$$2600 \times 32{\cdot}8/31{\cdot}8 = 2730 \text{ ml.}$$

Table 1. *Protocol of Exp.* 1

Time (min.)	Procedure
0	2600 ml. mixed sodium salts put in rumen. 2 ml. removed
5	*p*H 7·6
10	2 ml. *N* acetic acid added
15	*p*H 7·48. 2 ml. *N* acetic acid added
35	*p*H 7·57. 2 ml. *N* acetic acid added
40	2 ml. removed
45	*p*H 7·6
70	*p*H 7·6. 2 ml. *N* acetic acid added
80	*p*H 7·6. 2 ml. removed. 2 ml. *N* acetic acid added
100	*p*H 7·4
120	*p*H 7·5. 2 ml. removed. 83 ml. *N* acetic acid added
125	2 ml. removed
130	*p*H 5·82. 50 ml. *N* acetic added
145	*p*H 5·97. 40 ml. *N* acetic acid added
150	*p*H 5·78
160	*p*H 5·94. 40 ml. *N* acetic acid added. 2 ml. removed
170	*p*H 5·84
180	*p*H 5·9. 26 ml. *N* acetic acid added
185	*p*H 5·83
195	40 ml. *N* acetic acid added
200	*p*H 5·84. 21 ml. *N* acetic acid added. 2 ml. removed
220	*p*H 6·08. 50 ml. *N* acetic acid added. *p*H 5·75
240	*p*H 5·86. 2 ml. removed. Rumen contents 3800 ml.

The volume of the fluid in the rumen at the time at which the *p*H was changed was calculated in the following manner:

(1) Let V = the volume immediately before the change in *p*H

(2) and $V+Y$ the volume 5 min. after the addition of Y ml. of normal acetic acid;

then the total quantity of acid present before the change is

(3) $\frac{V}{100} \times C$ and the total quantity of acid present 5 min. after the change is

(4) $\left(\frac{V+Y}{100} \times C_1\right)$, where C is the concentration of acid in the sample taken before the change and C_1 is the concentration of acid 5 min. after the change.

V can be determined by the following equation:

(5) $$\left(\frac{V+Y}{100} \times C_1\right) - \left(\frac{V}{100} \times C\right) = Y.$$

Thus it was found that the volumes in the rumen are 2730 ml. at time 0, 3015 ml. at 120 min. and

3800 ml. at 240 min. These figures show that throughout the experiment there was an increase in the volume of the rumen contents, although the total amount of acid in the rumen was decreasing. of acid made to the rumen. From §§ (1) and (3) of this table the total acid in the rumen at various times can be calculated and is given in § (4) of this table. Then, from § (4) and from the percentage composi-

Table 2. *The results of analysis of rumen samples from sheep* 1, *at various times*

	Composition of dose	*p*H 7·5				*p*H 5·75			
Time (min.) ...		0	40	80	120	120	160	200	240
(1) Composition of rumen contents: ml. *N* acid/100 ml.	32·8	31·2	24·8	21·7	19·7	21·0	20·9	19·5	18·4
(2) Composition of rumen acid: % acetic	61·2	61·6	60·7	59·8	59·4	66·9	72·5	78·4	79·9
% propionic	21·9	21·7	21·2	21·8	22·4	17·6	15·0	11·7	11·7
% butyric	16·9	16·7	18·1	18·4	18·2	15·5	12·5	9·9	8·4
(3) Volume of rumen contents, ml.	2600	2730	2840	2920	3015	3100	3380	3610	3800
(4) Total acid in rumen, ml. *N* acid	853	853	705	634	594	675	706	704	700
(5) Additions to rumen, ml. *N* acetic acid	—	—	—	—		83	130	86	50
(6) Rumen contents, ml. *N* acid: Acetic	—	526	428	379	346	429	512	551	558
Propionic	—	176	149	139	130	119	106	83	82
Butyric	—	114	127	116·5	104	104	88	70	59
(7) Molar concentration of acids in rumen: Acetic	—	0·193	0·151	0·130	0·115	0·138	0·152	0·153	0·147
Propionic	—	0·065	0·053	0·048	0·043	0·0385	0·0315	0·023	0·0205
Butyric	—	0·053	0·046	0·040	0·035	0·0335	0·0260	0·0195	0·0155

Table 3. *The results of analysis of rumen samples from sheep* 4, *at various times*

	Composition of dose	*p*H 7·5	*p*H 5·65	
Time (min.) ...		0	120	240
(1) Composition of rumen contents: ml. *N* acid/100 ml.	33·4	31·1	28·8	27·8
sodium, g./100 ml.	0·72	0·67	0·61	0·51
(2) Composition of rumen acid: % acetic	41·1	41·3	40·2	61·4
% propionic	29·7	29·2	30·3	22·1
% butyric	29·2	29·5	29·5	16·5
(3) Volume of rumen contents, ml.	6310	6780	6780	7520
(4) Total acid in rumen, ml. *N* acid	2100	2100	1950	2090
(5) Additions to rumen, ml. *N* acetic acid	—	—	666*	
(6) Rumen contents, ml. *N* acid: Acetic	—	868	784	1280
Propionic	—	614	590	462
Butyric	—	620	575	345
(7) Molar concentration of acids in rumen: Acetic	—	0·128	0·116	0·170
Propionic	—	0·091	0·087	0·062
Butyric	—	0·092	0·085	0·046

* 300 ml. were added to produce the initial *p*H change, and the remainder at intervals to keep the *p*H constant.

This result is quite reasonable, for the rumen contents were hypertonic with respect to the blood. To calculate the volumes at intermediate times it has been assumed that the gain in volume was linear at each *p*H. These volumes are given in § (3) of Table 2: they are, of course, corrected for additions tion of the acid given in § (2), the total amounts of the individual acids in the rumen were calculated and are given in § (6). Finally, from the figures in § (6) and the volumes in § (3), the molar concentra tions of the individual acids are calculated and are given in § (7).

Table 4 describes a further experiment, essentially similar to those given in Tables 2 and 3 with the exception that it was started in the acid and not in the alkaline range as in previous experiments; the reaction was raised later to *p*H 7·5 by the addition of normal sodium hydroxide. Otherwise the experiment was conducted and the calculations were made in the same manner as given in Tables 2 and 3.

From these results the following points are apparent:

(1) When the rumen contents are at *p*H 7·5, though much acid is absorbed, the individual proportions of the acids change comparatively little; i.e. the acids are absorbed from the rumen roughly in proportion to their concentration.

(2) When the rumen contents are at about *p*H 5·8, the rate of loss is greater and the acids are lost in the order butyric > propionic > acetic.

(3) When the fluid in the rumen is at *p*H 7·5 the *p*H is relatively stable. Also, the sodium content of the rumen falls roughly in proportion to the loss of anions. This shows that the permeation of the rumen epithelium must be carried out by sodium ions and acetate anions in roughly equal proportions, and that anion exchange across the epithelium is not very important.

(4) When the rumen is at *p*H 5·8 the *p*H is very unstable and constant addition of acid is required to prevent drift to the vicinity of *p*H 7·0. It follows that much free acid must be lost from the rumen at this *p*H.

(5) The substances studied (fatty acid anions, sodium and water) are all moving from regions of higher to regions of lower concentration; thus the acid present in the blood is negligible compared with that in the rumen, while the sodium content of the blood (365 mg. % according to Dukes, 1943) is about half that of the rumen (720 mg. %).

Table 4. *The results of analysis of rumen samples from sheep 6, at various times*

	Composition of dose	*p*H 5·85				*p*H 7·5	
Time (min.) ...		0	40	80	120	120	240
(1) Composition of rumen contents:							
ml. *N* acid/100 ml.	33·3	30·3	26·6	23·2	20·5	—	15·9
sodium, g./100 ml.	0·7	0·58	0·55	0·54	0·49	0·51	0·42
(2) Composition of rumen acid:							
% acetic	43	43	50·1	57·4	61·5	—	65
% propionic	28·5	28·5	25·9	22·4	21·4	—	20·5
% butyric	28·5	28·5	24	21·4	17·1	—	15·5
(3) Volume of rumen contents, ml.	3200	3500	3680	3860	4005	3840	4090
(4) Additions to, and removals from, rumen:							
Removals in ml.	—	13	13	13	363	13	39
Addition of *N* acetic acid, ml.	—	82	84	54	60		—
Addition of *N* NaOH, ml.	—	—	—	—	120		—
(5) Total acid in rumen, ml. *N* acid, corrected for removals	1060	1060	984	902	830	—	744
(6) Rumen contents, ml. *N* acid, corrected for removals:							
Acetic	—	456	493	517	509	—	481
Propionic	—	302	255	202	177	—	153
Butyric	—	302	236	193	142	—	117
(7) Molar concentration of acids in rumen, corrected for removals:							
Acetic	—	0·130	0·134	0·134	0·127	—	0·106
Propionic	—	0·086	0·0695	0·0525	0·044	—	0·0335
Butyric	—	0·086	0·064	0·050	0·0355	—	0·0255

DISCUSSION

The operation of adding some hundreds of ml. of *N* acid to the rumen contents over a 2 hr. period, to keep the *p*H constant, provides a striking demonstration of the speed with which acid is absorbed from the rumen. This is even more interesting in that the *p*H at which this occurs (5·8) is not outside the normal limits, and that the rumen is cut off from the body except for its blood, nerve and lymphatic supply. This result shows that the need of understanding the mechanism involved in absorption from the rumen must be placed high. To ascertain this mechanism we must first calculate the relative permeabilities of the rumen to the different sub-

stances studied, and then consider the relationships of these relative permeability constants.

Let P_i = relative permeability of the rumen to the molecular species i; M_i = molecular weight of the species i; B_i = oil-water partition coefficient of the species i; x_i = number of CH_2 groups of the species i; R = gas constant per g.mol.; T = absolute temperature. Then it has been shown elsewhere that the following relationships hold (Danielli, 1941, 1943):

(i) If P_i does not vary with molecular species, permeation is by bulk flow of fluid, carrying all molecular species with it.

(ii) If $P_i M_i^{\frac{1}{2}}$ does not vary with molecular species, permeation is by simple diffusion through a homogeneous membrane or through a pore structure, the pores in which are large compared with the diameter of the largest molecule considered.

(iii) If $P_i M_i^{\frac{1}{2}}/B_i$ does not vary with molecular species, permeation is by a simple diffusion through a lipoid layer through which the molecules are penetrating rapidly.

(iv) If $P_i M_i^{\frac{1}{2}} \exp(2500x_i/RT)/B_i$ does not vary with molecular species, permeation is by simple diffusion through a lipoid layer into which the molecules penetrate slowly.

(v) If none of the above hold, but P_i for a given species is a function of concentration, secretory mechanisms are to be suspected.

Tables 5–7 show calculations made on the data provided by Tables 2–4 respectively. In the case of Table 5 the steps taken are:

(*a*) Calculation of ΔN, the amount of each acid lost from the rumen over each time interval. This is calculated from § (6) of Table 2.

(*b*) Calculation of $\bar{c}_T$, the average concentration of total acid (free acid + anion) in the rumen over the various time intervals. This is calculated from §§ (7) and (3) of Table 2.

(*c*) Calculation of $\bar{c}_A$, the average concentration of the different anions in the rumen over the various time intervals. This is obtained from (*b*) and from the equation $p\mathrm{H} = pK_a + \log [\text{salt}]/[\text{acid}]$. pK_a was taken as 4·73 for acetic, and as 4·8 for butyric and propionic acids.

(*d*) Calculation of $\bar{c}_{FA}$, the average concentrations of the free acids over the time intervals. This is obtained from the difference between (*b*) and (*c*).

(*e*) Calculation of P_A, the relative permeability to anions. At *p*H 7·5 the concentration of free acid is negligible, and the *p*H is stable. Hence only the anion is penetrating, and the amount of anion penetrating, ΔN_A, is given by ΔN_A of (*a*) above. Hence, P is given by $\Delta N_A/\bar{c}_A$.

(*f*) Calculation of $P_A M^{\frac{1}{2}}$, from the results given under (*e*). From §§ (*e*) and (*f*) of Table 5 it is seen that the values of P_A and of $P_A M^{\frac{1}{2}}$, at *p*H 7·5, are roughly constant for the three acids in any given time interval. The same is true of the similar sections of Tables 6 and 7. Oil-water partition coefficients for fatty acids increase by a factor of about 2·8-fold for each additional CH_2 group; e.g. if we take the partition coefficient for acetic acid as 1, that for propionic acid is $2{\cdot}8^{\frac{2}{3}} = 7{\cdot}8$. Calculation of $P_A M^{\frac{1}{2}}/B$ and of $P_A M^{\frac{1}{2}} \exp(2500x/RT)/B$ for the values at *p*H 7·5 shows that in neither of these cases is there any approximation to constancy for the three anions. Thus mechanisms (iii) and (iv) can be excluded. The experimental results are not sufficiently accurate, and the variation in molecular weight not sufficiently large, to distinguish between mechanisms (i) and (ii), though on the whole the evidence is in favour of (ii). In both (i) and (ii) the acid is lost from the rumen through water-filled pores, in the first case by bulk flow, and in the second by diffusion. There is no indication of the pores discriminating between molecules in virtue of their diameter, i.e. the pores must be large compared with the diameter of butyrate anion. We may therefore conclude that the lower fatty acid anions are lost from the rumen by passage through water-filled pores of diameter large compared with the diameter of butyric acid molecules, and that no other mechanism is of importance in the absorption of fatty acid anion.

In the first time interval of the experiment of Table 4, P is abnormally large: we suspect that this is due to shrinkage of the rumen epithelial cells caused by hypertonicity of the rumen contents, with concomitant enlargement of the porous area lying between the cells.

To analyse the results at *p*H 5·75, we shall assume, first, that the permeability of the pores is the same as at *p*H 7·5, and secondly that the permeation of the pores is by mechanism (ii): the results are in fact the same, within experimental error, whether (i) or (ii) is assumed to operate. Then in Table 5 the average value of $PM^{\frac{1}{2}}$ for the second and third time intervals is $2{\cdot}15 \times 10^3$. With this information we can carry out the following steps, recorded in Table 5:

(*g*) ΔN_A, the amount of anion lost through the pores at *p*H 5·75, is calculated from

$$\Delta N_A = 2{\cdot}15 \times 10^3 \bar{c}_A / M^{\frac{1}{2}}.$$

$\bar{c}_A$ is given in § (*c*) of Table 5.

(*h*) ΔN_{FA}, the amount of free acid lost through the pores at *p*H 5·75, is calculated from

$$\Delta N_{FA} = 2{\cdot}15 \times 10^3 \bar{c}_{FA} / M^{\frac{1}{2}}.$$

$\bar{c}_{FA}$ is given in § (*d*) of Table 5.

(*i*) This step involves the calculation of

$$\Delta N' = \Delta N - \Delta N_A - \Delta N_{FA},$$

i.e. the amount of acid lost by routes other than the water-filled pores. ΔN is given in § (*a*), ΔN_A in § (*g*) and ΔN_{FA} in § (*h*) of Table 5.*

* This calculation results in all the errors of experiment and calculation accumulating in the value obtained for $\Delta N'$, and so is a very stern test of the method.

Table 5. *Analysis of the results presented in Table 2*

Time interval (min.) ...	*p*H 7·5			*p*H 5·75		
	0–40	40–80	80–120	120–160	160–200	200–240
(*a*) ΔN = ml. *N* acid lost from rumen:*						
Acetic	98	49	33	47	48	48
Propionic	27	10	9	13	13	11
Butyric	17	10·5	10·5	19	15	11
Average concentration in rumen over the intervals:						
(*b*) $\bar{c}_T$: Total acetic	0·172	0·142	0·114	0·145	0·153	0·150
Total propionic	0·059	0·0505	0·0455	0·035	0·0275	0·022
Total butyric	0·0495	0·043	0·0395	0·0305	0·023	0·0175
(*c*) $\bar{c}_A$: Acetate anion	0·172	0·142	0·114	0·135	0·142	0·139
Propionate anion	0·059	0·0505	0·0455	0·032	0·025	0·020
Butyrate anion	0·0495	0·043	0·0395	0·028	0·021	0·016
(*d*) $\bar{c}_{FA}$: Free acetic acid	—	—	—	0·0102	0·0108	0·0106
Free propionic acid	—	—	—	0·0029	0·00235	0·0018
Free butyric acid	—	—	—	0·0025	0·0019	0·00145
(*e*) P_A = relative permeability to anion = $\frac{\Delta N_A}{\bar{c}_A} \times 10^{-2}$:						
Acetate	5·7	3·4	3·0	(Cannot be obtained directly from experimental results)		
Propionate	4·6	2·0	2·0			
Butyrate	3·5	2·3	2·5			
(*f*) $P_A M^{\frac{1}{2}} \times 10^{-3}$:						
Acetate	4·4	2·6	2·3	(Average value taken as $2{\cdot}15 \times 10^3$)		
Propionate	3·9	1·7	1·7			
Butyrate	3·2	2·1	2·3			
(*g*) ΔN_A = anion lost through pores $= \frac{2{\cdot}15 \times 10^3}{M^{\frac{1}{2}}} \bar{c}_A$ ml. *N* solution:						
Acetate	—	—	—	37·5	39·4	38·5
Propionate	—	—	—	8·1	6·3	5·1
Butyrate	—	—	—	6·6	5·0	3·8
(*h*) ΔN_{FA} = free acid lost through pores $= \frac{2{\cdot}15 \times 10^3}{M^{\frac{1}{2}}} \bar{c}_{FA}$ ml. *N* solution:						
Acetate	—	—	—	2·8	3·0	2·9
Propionate	—	—	—	0·7	0·6	0·5
Butyrate	—	—	—	0·6	0·5	0·3
(*i*) $\Delta N' = \Delta N - \Delta N_A - \Delta N_{FA}$ ml. *N* solution = acid lost other than through pores:						
Acetate	—	—	—	6·7	5·6	6·6
Propionate	—	—	—	4·2	5·9	5·4
Butyrate	—	—	—	11·8	10·5	6·9
(*j*) $P'_{FA} = \frac{\Delta N'}{\bar{c}_{FA}} \times 10^{-2}$ = relative permeability by non-porous route:						
Acetic	—	—	—	6·5	5·3	6·2
Propionic	—	—	—	14·5	25	30
Butyric	—	—	—	48	55	48
(*k*) $P'_{FA} M^{\frac{1}{2}} \times 10^{-3}$:						
Acetic	—	—	—	5·0	4·1	4·8
Propionic	—	—	—	12·5	21	25
Butyric	—	—	—	44	50	44
(*l*) $\frac{P'_{FA} M^{\frac{1}{2}}}{B} \times 10^{-3}$:						
Acetic	—	—	—	5·0	4·1	4·8
Propionic	—	—	—	4·5	7·5	9·0
Butyric	—	—	—	5·6	6·4	5·6

* Values from smoothed curves.

Table 6. *Analysis of the results presented in Table 3*

	*p*H 7·5	*p*H 5·65
Time interval (min.) ...	0–120	120–240
(*a*) ΔN = ml. *N* acid lost from rumen:		
Acetic	84	170
Propionic	24	132
Butyric	45	230
Average molar concentrations in the rumen over the intervals:		
(*b*) $\bar{c}_T$: Total acetic	0·122	0·160
Total propionic	0·089	0·073
Total butyric	0·089	0·064
(*c*) $\bar{c}_A$: Acetate anion	0·122	0·142
Propionate anion	0·089	0·065
Butyrate anion	0·089	0·057
(*d*) $\bar{c}_{FA}$: Free acetic acid	—	0·0176
Free propionic acid	—	0·0080
Free butyric acid	—	0·0070
(*e*) $P_A = \frac{\Delta N_A}{\bar{c}_A} \times 10^{-3}$ = relative permeability to anion:		
Acetic	6·9	(Cannot be obtained directly from experimental results)
Propionic	2·7	
Butyric	5·1	
(*f*) $P_A M^{\frac{1}{2}} \times 10^{-3}$:		
Acetic	5·3	(Value taken as $5{\cdot}3 \times 10^3$)
Propionic	2·3	
Butyric	4·7	
(*g*) ΔN_A = ml. *N* acid anion lost through pores = $\frac{5{\cdot}3 \times 10^3}{M^{\frac{1}{2}}} \bar{c}_A$:		
Acetic	—	97
Propionic	—	40·5
Butyric	—	33
(*h*) ΔN_{FA} = ml. *N* free acid lost through pores = $\frac{5{\cdot}3 \times 10^3}{M^{\frac{1}{2}}} \bar{c}_{FA}$:		
Acetic	—	12
Propionic	—	5
Butyric	—	4·1
(*i*) $\Delta N' = \Delta N - \Delta N_A - \Delta N_{FA}$ ml. *N* acid = acid lost other than through pores:		
Acetic	—	61
Propionic	—	86·5
Butyric	—	193
(*j*) $P'_{FA} = \frac{\Delta N'}{\bar{c}_{FA}} \times 10^{-3}$:		
Acetic	—	3·5
Propionic	—	10·8
Butyric	—	27·6
(*k*) $P'_{FA} M^{\frac{1}{2}} \times 10^{-4}$:		
Acetic	—	2·7
Propionic	—	9·2
Butyric	—	26·4
(*l*) $\frac{P'_{FA} M^{\frac{1}{2}}}{B} \times 10^{-4}$		
Acetic	—	2·7
Propionic	—	3·3
Butyric	—	3·4

Table 7. *Analysis of the results presented in Table 4*

	*p*H 5·85			*p*H 7·5
Time interval (min.) ...	0–40	40–80	80–120	120–240
(*a*) ΔN = ml. *N* acid lost from rumen over the intervals:				
Acetic	45	60	62	88
Propionic	47	53	25	24
Butyric	60	43	51	25
Average molar concentrations in rumen over the time intervals:				
(*b*) $\bar{c}_T$: Total acetic	0·132	0·134	0·131	0·117
Total propionic	0·078	0·0615	0·048	0·039
Total butyric	0·075	0·057	0·0425	0·0305
(*c*) $\bar{c}_A$: Acetate anion	0·123	0·125	0·122	0·117
Propionate anion	0·072	0·057	0·044	0·039
Butyrate anion	0·059	0·052	0·039	0·0305
(*d*) $\bar{c}_{FA}$: Free acetic acid	0·0093	0·0094	0·0092	—
Free propionic acid	0·0064	0·0050	0·0039	—
Free butyric acid	0·0062	0·0047	0·0035	—
(*e*) P_A = relative permeability to anion = $\frac{\Delta N_A}{\bar{c}_A} \times 10^{-2}$:				
Acetate	(Cannot be obtained directly from experimental results.)			7·5
Propionate				6·2
Butyrate				8·1
(*f*) $P_A M^{\frac{1}{2}} \times 10^{-3}$:				
Acetate	(Average value for a 40 min. period taken as $2{\cdot}06 \times 10^3$.)			5·8
Propionate				5·3
Butyrate				7·4
(*g*) ΔN_A = ml. *N* anion lost through pores = $\frac{2{\cdot}06 \times 10^3}{M^{\frac{1}{2}}} \bar{c}_A$:				
Acetate	32·7	33·2	32·4	—
Propionate	17·4	13·8	10·7	—
Butyrate	13·2	11·6	8·7	—
(*h*) ΔN_{FA} = ml. *N* free acid lost through pores = $\frac{2{\cdot}06 \times 10^3}{M^{\frac{1}{2}}} \bar{c}_{FA}$:				
Acetic	2·5	2·5	2·5	—
Propionic	1·6	1·2	1·0	—
Butyric	1·4	1·1	0·8	—
(*i*) $\Delta N' = \Delta N - \Delta N_A - \Delta N_{FA}$ ml. *N* acid = acid lost other than through pores:				
Acetic	9·8	24·3	27·1	—
Propionic	28·0	38·0	13·3	—
Butyric	51·4	30·3	41·5	—
(*j*) $P'_{FA} = \frac{\Delta N'}{\bar{c}_{FA}} \times 10^{-3}$:				
Acetic	1·05	2·6	2·9	—
Propionic	4·4	7·6	3·4	—
Butyric	8·3	6·5	11·9	—
(*k*) $P'_{FA} M^{\frac{1}{2}} \times 10^{-3}$:				
Acetic	8·2	20	22	—
Propionic	37	64	29	—
Butyric	76	60	109	—
(*l*) $\frac{P'_{FA} M^{\frac{1}{2}}}{B} \times 10^{-3}$:				
Acetic	8·2	20	22	—
Propionic	13	23	10	—
Butyric	9·8	7·8	15·3	—

Since we have already shown that fatty acid anion is lost in significant amounts through water-filled pores only, $\Delta N'$ must consist of free acid lost by mechanisms other than passage through water-filled pores. The nature of the mechanisms of the loss of this free acid $\Delta N'$ can be examined in the following steps, as in Table 5:

(*j*) Calculate P'_{FA} from $P'_{FA} = \Delta N'/C_{FA}$, $\Delta N'$ being given by § (*i*) and $\bar{c}_{FA}$ by § (*d*) of Table 5.

(*k*) From (*j*), calculate $P'_{FA}M^{\frac{1}{2}}$.

(*l*) From (*k*), calculate $P'_{FA}M^{\frac{1}{2}}/B$.

Inspection of the results shown in Table 5 indicates that, whereas there are wide variations in $P'_{FA}M^{\frac{1}{2}}$, $P'_{FA}M^{\frac{1}{2}}/B$ is constant within experimental error for these three acids. The values of

$$P'_{FA}M^{\frac{1}{2}} \exp(2500x/RT)/B$$

also show wide variations (the values of this are not shown in Table 5). The same is true of the results of the experiments of Tables 6 and 7. Consequently the conclusion holds that at *p*H 5·75 free fatty acid represented by N' leaves the rumen by mechanism (iii), i.e. by simple diffusion through a lipoid layer, and that this diffusion is rapid. This loss of acid is, of course, in addition to free acid lost by diffusion through the water-filled pores, and to the anion lost by diffusion through the water-filled pores.

The general conclusion reached is that material may move through the rumen epithelium by two routes, one route involving passage through water-filled pores, and the other diffusion through a lipoid membrane. We have made no experiments to determine the nature of the pores, etc., but it seems probable that they are pores in the cement lying between the cells of the rumen epithelium, and that the lipoid membrane involved is that of the epithelial cells themselves. If this is so, the permeability of the rumen epithelium must in some respects resemble that of the capillary endothelium, where these two alternative routes for penetration (through intercellular pores, and through cell membranes) are also available (Danielli & Stock, 1944).

Experiments of greater accuracy are needed to determine the exact mechanism of the loss of material through the water-filled pores, and to determine how far the pressure in the rumen and electro-osmotic effects are of importance.

*The control of p*H *and the rate of absorption from the normally filled rumen*

The comparative stability of the *p*H of the rumen contents has hitherto been attributed to the continual inflow of alkaline saliva. It is clear from these experiments that this is only partially true, for in addition to the inflow of saliva, the *p*H of which is in the region of 8, the rapid absorption of free acid plays a part in regulating the *p*H of the ingesta of the rumen. Whether or not the latter mechanism is in itself sufficient to maintain the *p*H within physiological limits in the absence of salivary flow can only be decided by further research.

In Fig. 1 the influence of *p*H on the quantity of volatile acid absorbed from the normally filled rumen is illustrated. The concentrations of volatile acid upon which the calculations are made were average figures taken from three sheep fed upon hay and three other sheep allowed to graze freely on young summer grass (Phillipson, 1942). In the former case the average figure was 0·095 *N* fatty acid and in the latter case the average figure was 0·135 *N* fatty acid.

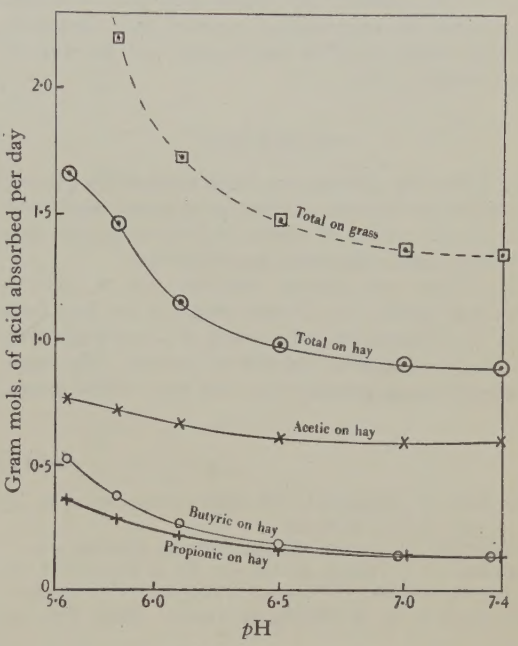

Fig. 1. The effect of variation in rumen *p*H and in diet on the daily absorption of fatty acid from the rumen of sheep.

The points on these curves were calculated by the following steps:

(1) All pK_a were assumed to be 4·8.

(2) The percentages of anion and acid present in the rumen at various *p*H values were calculated.

(3) The concentrations of anion and of free acid in the rumen were calculated.

(4) The losses of total acid from the rumen were calculated from (3) and from the values of the permeabilities obtained in this work.

The average *p*H of the ingesta of the rumen of sheep eating hay was 6·45 while that of sheep eating grass was 6·2, which further accentuates the difference between the rates of absorption in the two groups of sheep as a result of the differences in the proportion of free fatty acid at the two *p*H values. At the two values given the absorption of acid in sheep at grass exceeded that of the sheep on hay by two-thirds.

The variation in *p*H of the rumen contents of sheep at grass was found to be 6·70–5·44; at all concentrations of volatile acid the rate of absorption is doubled over this range on passing from the extreme alkaline to the extreme acid figure. This is interesting, for in previous work no simple relation could be found between the rate of absorption of volatile acid from the rumen and the concentration of volatile acid in the rumen (Barcroft *et al.* 1944). These animals were taken straight from pasture and the *p*H of the rumen contents was not determined; variation in this factor would produce large variations in the quantity of volatile acid absorbed, and thus mask the relationship between the concentration of volatile acid in the rumen and the rate of absorption of acid.

SUMMARY

The following conclusions were reached by placing a solution of sodium acetate, propionate and butyrate in the rumen of sheep and studying the rate at which these substances are absorbed:

(1) When the rumen contents are at *p*H 7·5, fatty acid anion only is absorbed, i.e. no free fatty acid is absorbed, and the anion is accompanied by a roughly equivalent amount of sodium. The anion is absorbed by passage through water-filled pores, the diameter of which is large compared with butyric acid. These pores probably lie in the intercellular cement of the rumen epithelium.

(2) When the rumen contents are at *p*H 5·8, free acid is lost from the rumen in large amounts in addition to the loss of fatty acid anion. Part of this free acid is absorbed by passage through water-filled pores, but the greater part of it passes through the lipoid membranes of the epithelial cells.

(3) In consequence of the difference in the two mechanisms, at alkaline *p*H the substances are lost in the order acetate > propionate > butyrate, whereas at acid *p*H the order is butyrate > propionate > acetate.

(4) All the substances studied (fatty acid anions, sodium and water) move from regions of higher to regions of lower concentration.

(5) The total fatty acid absorbed from the rumen of a sheep on pasture grass may readily be twice that absorbed from the rumen by the same sheep on hay.

(6) The permeability of the rumen epithelium is such that the *p*H of the ingesta tends to move towards neutrality, independently of the neutralizing action of the saliva.

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